UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

APLICACIÓN DE TÉCNICAS MOLECULARES AVANZADAS PARA LA EVALUACIÓN DE MECANISMOS DE ACCIÓN DE BACTERIAS PROBIÓTICAS FRENTE AL ESTRÉS OXIDATIVO

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A mis padres,

a Juanma

INDICE

RESUMEN

Determinados alimentos, como los sometidos a procesos tecnológicos severos, proporcionan a la dieta compuestos procedentes de la oxidación de lípidos (malondialdehído, MDA) y de proteínas (ácido aminoadípico, AAA) que presentan un potencial efecto perjudicial, como la alteración del estado redox celular, que lleva a la aparición de estrés oxidativo. Se sabe que estos compuestos causan daño oxidativo en las células intestinales, estando relacionado dicho estrés con patologías del tracto gastrointestinal (TGI), como la colitis ulcerosa, la enfermedad de Crohn y otras enfermedades inflamatorias del intestino. Para contrarrestar los efectos negativos de la ingesta de productos oxidados se han propuesto estrategias antioxidantes en los alimentos, entre las que se encuentran la suplementación con compuestos fenólicos, presentes de manera natural en frutas y verduras, o con bacterias probióticas, que han demostrado tener efecto protector en el TGI. El efecto beneficioso de las bacterias probióticas responde a mecanismos moleculares poco conocidos, ya que se desconoce si estos microorganismos tienen capacidad para detoxificar los productos de oxidación procedentes de la dieta, o cómo se producen a nivel molecular las sinergias entre probióticos y compuestos fenólicos. Para progresar en este campo y entender los procesos fisiológicos y rutas metabólicas de las bacterias probióticas, se requiere la aplicación de técnicas avanzadas en el campo de la biología molecular, como la PCR en tiempo real, la citometría de flujo o el análisis del proteoma bacteriano, con los que se puede obtener información valiosa para la comprensión de los efectos beneficiosos de compuestos fenólicos y bacterias probióticas. En la presente Tesis, dos bacterias potencialmente probióticas, *Lactobacillus reuteri* y *Enterococcus faecium*, han sido expuestas al efecto de MDA, AAA y peróxido de hidrógeno en distintas concentraciones además de combinar la exposición a este último con un compuesto fenólico (ácido clorogénico). Se ha visto que los compuestos prooxidantes producen especies reactivas del oxígeno (ROS), promueven la oxidación de lípidos y proteínas bacterianas e inducen la puesta en marcha de mecanismos de defensa frente al estrés oxidativo. Por su parte el compuesto fenólico estimula la carbonilación de proteínas, mediando en la señalización celular que conduce a activar respuestas antioxidantes. Las técnicas avanzadas utilizadas en la presente Tesis Doctoral han permitido conocer de forma precisa los mecanismos de expresión génica y síntesis proteica que se activan en bacterias probióticas frente a la agresión de productos de oxidación procedentes de la dieta.

Palabras Clave: *L. reuteri*; *E. faecium*; estrés oxidativo; expresión génica; citometría flujo; proteómica.

ABSTRACT

Certain foods, such as those subjected to severe technological processes, provide the diet with lipid (malondialdehyde, MDA) and protein (aminoadipic acid, AAA) oxidation products. These compounds display potential harmful effects, such as the alteration of the cellular redox state, which leads to the appearance of oxidative stress. These compounds are known to cause oxidative damage in intestinal cells and such stress is related to pathologies of the gastrointestinal tract (GIT), such as ulcerative colitis, Crohn's disease and other inflammatory bowel diseases. To counteract the negative effects of the intake of oxidized products, antioxidant strategies have been proposed in food, among which are the supplementation with phenolic compounds, naturally present in fruits and vegetables, and probiotic bacteria, which are known to extert a protective effect in the GIT. The beneficial effect of probiotic bacteria responds to little-known molecular mechanisms, since it is unknown if these microorganisms have the capacity to detoxify oxidation products from the diet, or how synergies between probiotics and phenolic compounds occur at the molecular level. To progress in this field, understanding the physiological processes and metabolic pathways of probiotic bacteria, requires the application of advanced techniques of molecular biology, such as real-time PCR, flow cytometry or bacterial proteome analysis, with which valuable information can be obtained for the understanding of the beneficial effects of phenolic compounds and probiotic bacteria. In this Doctoral Thesis, two potentially probiotic bacteria, *Lactobacillus reuteri* and *Enterococcus faecium*, have been exposed to MDA, AAA and hydrogen peroxide at different concentrations in addition to combining the exposure to the latter with a phenolic compound (chlorogenic acid). Pro-oxidant compounds have been shown to produce reactive oxygen species (ROS), promote oxidation of bacterial lipids and proteins and induce the activation of bacterial antioxidant responses. On the other hand, the phenolic compound stimulates protein carbonylation and cell signaling, and hence, enhancing the antioxidant responses. The advanced techniques used in this Doctoral Thesis have allowed gaining precise information on the mechanisms of gene expression and protein synthesis that are activated in probiotic bacteria against the damage caused by dietary oxidation products.

Keywords: *L. reuteri*; *E. faecium*; oxidative stress; gene expression; flow cytometry; proteomics.

1. INTRODUCCIÓN

1. INTRODUCCIÓN

1. 1. Estrés oxidativo

1.1.1. Concepto, causas y consecuencias

El oxígeno es esencial para la supervivencia de la vida aerobia e interviene en reacciones de gran importancia como la reacción de oxidación, un proceso bioquímico en el que se produce la pérdida de electrones. Esta reacción va unida a otra de ganancia de electrones que se denomina reducción. El intercambio de electrones que se ocasiona en estas reacciones de oxidación-reducción (redox) es el mecanismo mediante el que se produce la obtención de energía en las células (Kohen & Nyska, 2002).

En las reacciones redox se produce la formación de radicales libres, especies químicas que tienen un electrón desapareado en su orbital más externo, condición que confiere una elevada capacidad de reacción debido a la búsqueda de la estabilidad por parte de la molécula. Los radicales libres se producen continuamente en las células: como subproductos accidentales del metabolismo; deliberadamente durante procesos como la fagocitosis (Cheeseman & Slater, 1993), o como respuesta celular a xenobióticos (Valko et al., 2006), citoquinas o invasión bacteriana (Finkel & Holbrook, 2000).

Los radicales libres son conocidos por tener una actividad doble en los sistemas biológicos. Por un lado, pueden tener efectos beneficiosos al actuar como señales celulares en la respuesta fisiológica a tóxicos (Valko et al., 2006). Por otro lado, en altas concentraciones pueden reaccionar con moléculas biológicas y producir cambios en la composición química, oxidándolas, o en la estructura celular, que pueden llevar a la muerte celular o a daños en tejidos biológicos. Este segundo efecto, con consecuencias negativas para la homeostasis y supervivencia celular, se conoce como estrés oxidativo (Valko et al., 2006).

Los radicales libres se pueden formar a partir de muchas moléculas, pero los de mayor importancia, por el daño que producen, son los derivados de la molécula de oxígeno, que se generan de manera endógena durante el metabolismo oxidativo de la mitocondria, peroxisomas y activación de células inflamatorias, y de manera exógena mediante agentes ambientales que inducen su formación (Valko et al., 2006).

Las sustancias reactivas del oxígeno (en inglés, *reactive oxygen species*, ROS) incluyen iones de oxígeno, radicales libres y peróxidos. Es necesario tener que cuenta que, si bien todos los radicales libres son considerados ROS, hay ROS que no son radicales libres (no poseen electrones desapareados), pero igualmente promueven la oxidación. La reducción del oxígeno molecular (O_2) da lugar al ion superóxido (O^-_2), que es el precursor de la mayoría de ROS, como el peróxido de hidrógeno (H_2O_2), que a su vez

puede descomponerse en presencia de metales de transición para generar el radical hidroxilo (OH^-), que reacciona con los componentes celulares (Valko et al., 2007).

Por su parte los derivados de la molécula de nitrógeno, como el óxido nítrico (NO^-), que se forma en los tejidos mediante sintasas de óxido nítrico específicas; el dióxido de nitrógeno ($NO_2^{\bullet}$) y compuestos no radicales como el peroxinitrito ($ONOO^-$) y el trióxido de dinitrógeno (N_2O_3), son igualmente reactivos y peligrosos desde un punto de vista biológico y están implicados en una gran variedad de enfermedades (Battacharyya et al., 2014)

Para prevenir la formación de radicales libres o limitar sus efectos dañinos las células han desarrollado una variedad de defensas antioxidantes endógenas. Estas incluyen enzimas que descomponen peróxidos, proteínas que secuestran metales de transición o una variedad de compuestos que eliminan radicales libres (Cheeseman & Slater, 1993). En condiciones fisiológicas, estos mecanismos de defensa antioxidante neutralizan las ROS y nitrógeno y, por lo tanto, sus consecuencias negativas. Sin embargo, en determinadas circunstancias (contaminación, tabaco, ejercicio físico intenso, procesos patológicos o ingesta de alimentos oxidados) se puede producir un desequilibrio entre la cantidad de sustancias prooxidantes existentes en el medio y las defensas antioxidantes, que da lugar al ya descrito, estrés oxidativo, proceso en el que se pueden provocar daños en moléculas de importancia biológica por parte de radicales libres y otras moléculas prooxidantes (Finkel & Holbrook 2000) (Figura 1.1). Las consecuencias del daño oxidativo sobre lípidos, proteínas y ADN pueden desencadenar daños celulares permanentes, así como desequilibrio en procesos fisiológicos, induciendo enfermedades (Zhang et al., 2019).

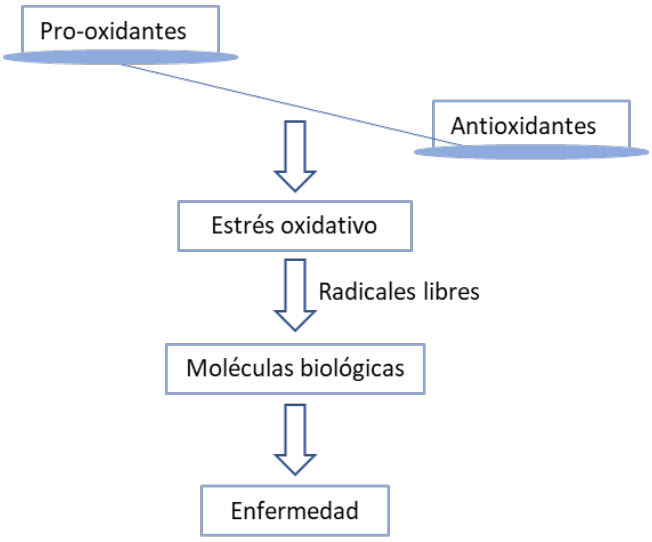

Figura 1.1. Esquema de mecanismo de aparición del estrés oxidativo.

Los daños celulares provocados por el estrés oxidativo tienen consecuencias sobre la salud animal como el aumento de la probabilidad de sufrir infecciones o parasitosis, así como alteraciones en los parámetros productivos y alteraciones en la calidad de la carne (Estévez, 2015; Soladoye et al., 2015). Además, en humanos, el estrés oxidativo se ha relacionado con la predisposición a sufrir diversas enfermedades, entre las que se encuentran algunas del tracto gastrointestinal como enfermedad de Crohn, colitis ulcerosa, cáncer colorrectal y diabetes (Tian et al., 2017).

1.1.2. Estrés oxidativo y enfermedad

El daño oxidativo producido por los radicales libres tiene un efecto acumulativo en biomoléculas como ADN, proteínas y lípidos. Estos daños juegan un papel importante en el desarrollo de enfermedades como algunos tipos de cáncer, aterosclerosis, artritis o enfermedades neurodegenerativas (Halliwell & Gutteridge, 1999).

Se ha visto que el estrés oxidativo está implicado en diversas afecciones patológicas que incluyen enfermedades cardiovasculares, cáncer, trastornos neurológicos, diabetes, isquemia/reperfusión o envejecimiento (Dhalla et al., 2000; Jenner, 2003; Dalle-Donne et al., 2006; Sayre et al., 2012). El proceso de envejecimiento se debe a las consecuencias de la acción de los radicales libres mediante la peroxidación de lípidos, daño en el ADN u oxidación de proteínas (Harman, 1956).

Las enfermedades derivadas de la acción de los radicales libres se dividen en dos grupos: el primero incluye las enfermedades producidas por el estrés oxidativo mitocondrial, que modifica la tolerancia a la glucosa (cáncer y diabetes mellitus); en el segundo grupo se encuentran enfermedades caracterizadas por producir inflamación (aterosclerosis, inflamación crónica, isquemia y reperfusión) (Valko et al., 2007).

Entre las enfermedades inflamatorias se encuentran las del intestino (en inglés, *Inflamatory bowel diseases*, IBD), que están caracterizadas por ser crónicas, idiopáticas y complejas. En ellas están incluidas la enfermedad de Crohn y la colitis ulcerosa. Estas patologías se describen como una inflamación crónica del intestino delgado o el colon, causadas por una respuesta inmune no regulada, al producirse la infiltración en la mucosa de células fagocíticas activadas que generan ROS y especies reactivas de nitrógeno (en inglés *reactive nitrogen species*, RNS) se altera la homeostasis celular por el daño en macromoléculas que contribuye al deterioro celular y aumenta la permeabilidad de la mucosa, acelerando y perpetuando la inflamación en curso. Además, la mucosa intestinal está permanentemente expuesta a la acción pro-oxidante de los productos de la oxidación proteica y

lipídica (estrés oxidativo luminal) que puede contribuir a un desajuste del estado redox del intestino (Estévez & Luna, 2017).

En la enfermedad de Crohn la inflamación se produce en cualquier parte del tracto gastrointestinal, pudiendo ocurrir de forma segmentaria y es transmural, afectando a todas las capas de la pared intestinal. Se ha visto un aumento de la actividad xantina oxidasa y Mn-superóxido dismutasa (SOD) en la mucosa inflamada de pacientes con enfermedad de Crohn (Kruidenier et al., 2003), así como un aumento del estrés oxidativo y una disminución de los antioxidantes, volviendo a niveles normales en la mejora de la condición del paciente (Maor et al., 2008.).

Por su parte, en la colitis ulcerosa la inflamación suele darse en el colon, empezando en el recto y avanzando en sentido proximal a medida que avanza la enfermedad, con inflamación continua que afecta solo a la capa mucosa de la pared intestinal. Se conoce que un defecto en las defensas antioxidantes de la mucosa favorece la aparición de la enfermedad (Bhattacharyya et al., 2014).

Los pacientes con enfermedad de Crohn y colitis ulcerosa tienen mayor riesgo de desarrollar cáncer colorrectal (CCR). El estrés oxidativo en el tejido inflamado puede favorecer la aparición de tumores malignos (Roessner et al., 2008) y el óxido nítrico (Sawa & Ohshima, 2006) y la peroxidación lipídica (Bartsch & Nair, 2005) pueden contribuir a la patogenia del cáncer colorrectal (CCR). Sin embargo, en esta patología también intervienen otros factores, como los genéticos (Marley & Nan, 2016).

La inflamación que se produce en estas enfermedades va unida a la formación de ROS y metabolitos de nitrógeno; sin embargo, la implicación que estos compuestos oxidantes tiene sobre el origen y desarrollo de la enfermedad o sobre la modulación de su evolución, todavía no está definida, debido a que es difícil determinarlos por la corta vida media que tienen los radicales (Cheeseman & Slater, 1993).

Para prevenir el daño producido por los radicales libres, se activan las defensas antioxidantes de la mucosa intestinal, donde en primer lugar la enzima SOD convierte O_2^- en H_2O_2, que es neutralizado a H_2O por las enzimas catalasa (CAT) o glutatión peroxidasa (GPox). Por otro lado, Tapia & Araya (2006), han señalado una disminución en los niveles de los antioxidantes más relevantes de la mucosa detectada en pacientes con estas afecciones intestinales.

1.2. Oxidación de la carne

El estrés oxidativo tiene lugar en el animal vivo; sin embargo, tras el sacrificio y debido a una serie de cambios que suceden en el músculo esquelético como la bajada del pH, el colapso de las defensas

antioxidantes y la exposición de la canal al oxígeno ambiental, las reacciones de oxidación no solo se mantienen activas en la carne *post-mortem*, sino que se acentúan (Bekhit et al., 2013). Estas reacciones, que tienen lugar durante el almacenamiento en frío de la carne (refrigeración/congelación), se potencian durante el procesado debido a la reducción de tamaño de las piezas y manipulación, adición de sal y otros ingredientes prooxidantes, aumento de las temperaturas y aplicación de muchos otros procesos (radiación, altas presiones hidrostáticas, etc.) que se ha demostrado que favorecen la oxidación de lípidos y proteínas en la carne (Soladoye et al., 2015).

Los cambios oxidativos que se producen en lípidos y proteínas de la carne suponen la degradación de las estructuras esenciales, conduciendo al desarrollo de olores y sabores anómalos, así como cambios en la textura o en la capacidad de retención de agua de las proteínas. Además, se puede producir la formación de compuestos tóxicos y la pérdida de nutrientes (Matissek et al., 1998; Contini et al., 2014). Esta es la causa principal, no dependiente de microorganismos, de deterioro de la carne durante su procesado (Xiao et al., 2013), haciendo que disminuya el valor de ésta y el de sus productos en el mercado (Sampels, 2013).

Finalmente, hay estudios que han sugerido que la susceptibilidad de la carne a la oxidación puede estar también relacionada con la raza y la especie del animal, el tipo de músculo, la situación anatómica del mismo, la dieta consumida por los animales y la selección genética (Min et al., 2008; Soladoye et al., 2015; Xiong & Guo, 2020). Además, los iones metálicos y los metales pesados presentes en las enzimas y metaloproteínas o los procedentes de máquinas de procesado, ya sea por roce o debido a la disolución ácida de metales de los factores superficiales, podrían promover la oxidación en la carne (Rulíšek & Vondrášek, 1998; Jacobsen et al., 2008). La exposición de la carne y de los productos cárnicos al oxígeno, la luz y la temperatura y los métodos de conservación, como la refrigeración, la congelación o los tratamientos tecnológicos, como la adición de aditivos, térmicos, las altas presiones y los envasados, también podrían influir en el grado de oxidación (Falowo et al., 2014).

1.2.1 Oxidación en lípidos

Los lípidos en la carne se encuentran ampliamente distribuidos, a nivel intracelular y extracelular, en forma de triglicéridos, fosfolípidos y esteroles, que son muy sensibles a la oxidación debido a su inestabilidad, sobre todo en la manipulación post-mortem y en el almacenamiento de la carne (Bekhit et al., 2013). Las consecuencias de la oxidación de lípidos son, entre otras, desarrollo de olor a rancio y sabores anómalos, decoloración, pérdida de valor nutritivo, reducción de la vida útil y acumulación de compuestos tóxicos, que podrían llegar a ser perjudiciales para la salud de los consumidores (Richards et al., 2002; Chaijan, 2008; Mapiye et al., 2012).

1. INTRODUCCIÓN

La oxidación lipídica se inicia en la fracción fosfolipídica de las membranas celulares, al ser rica en ácidos grasos poliinsaturados (Monahan et al., 1993; Buckley et al., 1995) y tiene lugar como una reacción en cadena que comienza cuando los radicales libres extraen un átomo de hidrógeno de una molécula de ácido graso, que da como resultado un radical lipídico (R*). Este radical, reacciona con el oxígeno molecular formando un radical peroxi (ROO*), que sustrae un átomo de hidrógeno a otro ácido graso, formando un hidroperóxido (ROOH) y un nuevo radical libre (R*), y propaga la reacción de oxidación. Los hidroperóxidos pueden formar radicales oxi (RO*) e hidroxilo (RH*) que pueden iniciar ambas oxidaciones. La reacción termina con la formación de productos menos reactivos (Figura 1.2).

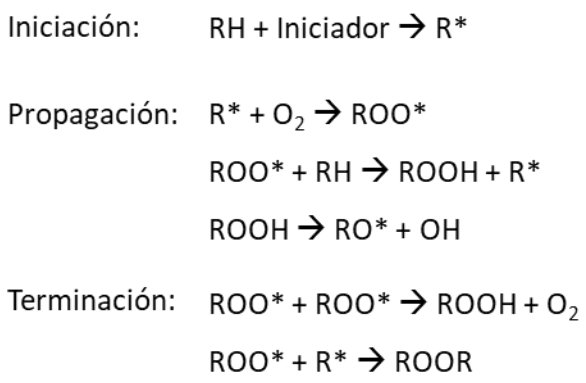

Figura 1.2. Esquema de oxidación lipídica (Fuente: Frankel et al., 1994).

Como consecuencia de estas reacciones, por la descomposición de los hidroperóxidos con metales divalentes (hierro y cobre), se forman productos secundarios de la peroxidación, como el malondialdehído (MDA) (Kanner, 1994), que es mutagénico en bacterias y células de mamíferos y carcinogénico en ratas (Valko et al., 2007). El MDA generalmente es el compuesto utilizado para medir el grado de oxidación lipídica. La formación de aldehídos está directamente relacionada con la degradación del olor y sabor de la carne, así como de la estabilidad y funcionalidad de sus proteínas (Lynch et al., 2001; Min & Ahn, 2005). Finalmente, es ampliamente reconocido que el MDA es un producto tóxico, capaz no solo de alterar la funcionalidad de proteínas y procesos biológicos en tejidos y órganos en animales y humanos, sino que además puede unirse al ADN, provocando mutaciones que se han descrito como la base molecular del desarrollo de algunos tipos de cánceres, incluido el CCR (Valko et al., 2004).

1.2.2 Oxidación en proteínas

Las proteínas, al ser componentes del tejido muscular, tienen un papel determinante en la carne y en los productos cárnicos desde el punto de vista sensorial, nutricional y tecnológico (Estévez et al., 2011;

Matarneh et al., 2017). Como consecuencia de los procesos de desnaturalización, proteolisis y oxidación de las proteínas de la carne, se producen cambios en su calidad, en las características de textura, color, aroma, sabor, capacidad de retención de agua y funcionalidad biológica (Falowo et al., 2014). El tejido muscular está compuesto principalmente por proteínas musculares, que han sido objeto de numerosos estudios referentes a las modificaciones producidas en la carne y en los productos cárnicos (Toldrá, 1998; Van Laack et al., 2000; Kemp et al., 2010; Kazemi et al., 2011), siendo la oxidación de proteínas uno de los procesos identificados como clave en el deterioro de la calidad sensorial, tecnológica y nutritiva de la carne y los derivados cárnicos (Lund et al., 2011).

La oxidación proteica, en la que se produce la modificación covalente de las proteínas, puede estar inducida por factores físicos: luz e irradiación (Dalsgaard et al., 2012; Wang et al., 2015); o por especies químicas como radicales, metales de transición, pigmentos hemo o azúcares reductores (Frederiksen et al., 2008; Estévez & Heinonen, 2010; Villaverde & Estévez, 2013; Chen et al., 2016).

Los radicales libres que producen una oxidación directa de las proteínas incluyen a ROS, RNS y especies reactivas del cloro (RCS). También pueden intervenir productos secundarios de la oxidación lipídica, como el MDA.

Las dianas de las ROS son la cadena principal del péptido y los grupos funcionales situados en la cadena lateral de los residuos de aminoácidos (Estévez, 2015). Sin embargo, las rutas de oxidación y la naturaleza de los productos finales dependen de la diana, el oxidante y la intensidad de las condiciones prooxidantes del medio (Davies, 2005). Como consecuencia del daño oxidativo en proteínas se producen alteraciones como las interacciones proteína-proteína (polimerización o formación de agregados), la escisión del péptido o modificaciones en cadenas laterales de los aminoácidos (Xiong, 2000; Stadman & Levine, 2003; Lund et al., 2011). El mayor daño producido por parte de las ROS es en estructuras con dobles enlaces, estructuras aromáticas y aminoácidos azufrados, como metionina y cisteína.

Entre los aminoácidos susceptibles de ser oxidados se encuentra la lisina que, al oxidarse, da lugar al semialdehído aminoadípico (AAS), que constituye un producto intermediario de la oxidación, siendo el producto final de ésta el ácido aminoadípico (AAA, Estévez & Luna, 2017). Se ha demostrado que la ingesta de AAA en animales de experimentación tiene efectos adversos (Akagawa et al., 2005; Sell et al., 2007; Estevez et al., 2009; Timm-Heinrich et al., 2013). Además, se ha visto que el AAA, en concentraciones existentes en los alimentos (200 µM), puede producir alteraciones mitocondriales, estrés oxidativo, apoptosis y necrosis en células pancreáticas intestinales humanas y de ratones (Díaz-Velasco et al., 2020; Estaras et al., 2020). La alteración de la microbiota intestinal se ha visto tanto *in*

vitro (Arcanjo et al., 2019) como *in vivo* (Goethals et al., 2020), producida por la ingesta de proteínas y aminoácidos oxidados.

Las RNS, como los radicales $NO^{\bullet}$ (óxido nítrico) y el $NO_2^{\bullet}$ (dióxido de nitrógeno) y los no radicales como el HNO_2 (ácido nitroso) y el ONOOH (ácido peroxinitroso), provocan la nitración de aminoácidos, siendo esta reacción irreversible y que puede afectar a la regulación de la señalización celular y a enzimas metabólicas (Halliwell & Gutteridge, 1999).

Entre las principales consecuencias de la oxidación de proteínas en carne y derivados cárnicos se encuentran: i) pérdida de la funcionalidad proteica y su capacidad para formar emulsiones, geles, retener agua en la carne, etc. (Soladoye et al., 2015); ii) alteración de la textura de la carne como consecuencia de la formación de enlaces cruzados y alteración del proceso de tenderización postmortem (Lund et al., 2011; Soladoye et al., 2015); iii) pérdida de valor nutritivo debido a la oxidación de aminoácidos esenciales y a la difícil digestibilidad de proteínas oxidadas (Soladoye et al., 2015) y iv) posibles efectos tóxicos en el consumidor como consecuencia de la ingesta de proteínas y aminoácidos oxidados (Estévez & Xiong, 2019).

1. 3. Ingesta de carne oxidada, estrés oxidativo y salud

Uno de los principales factores que influyen en el estado redox, tanto en humanos como en animales, es la composición de la dieta (Serafini & Del Rio, 2004). Estudios recientes destacan que, tras la ingesta de alimentos, se producen reacciones oxidativas en las primeras fases de la digestión, que llevan a la formación de productos de oxidación con potencial tóxico (Van Hecke et al., 2015., Van Hecke et al., 2016). Estos compuestos al ser captados por el tracto gastrointestinal se distribuyen por la sangre, llegando a los demás órganos (Estévez & Luna, 2016).

Las principales causas de estrés oxidativo descritas en animales son la ingesta de piensos con grasas oxidadas y micotoxinas y la exposición a temperaturas elevadas (Estévez, 2015). En el caso de los humanos, la fuente de estrés oxidativo dietético es la ingesta de lípidos y proteínas oxidadas, siendo los alimentos de origen animal (carne y productos cárnicos) los que presentan un mayor contenido en ellos (Soladoye et al., 2015; Estévez & Xiong, 2019).

Ciertos alimentos, tras su ingesta, favorecen la aparición de estrés oxidativo postprandial siendo la toma sostenida de carne roja y productos lácteos, lo que produce un aumento de marcadores de oxidación en plasma de humanos y animales (Van Hecke et al., 2016; Jakobsen et al., 2017). Los motivos por los que estos alimentos son inductores de estrés oxidativo son, principalmente, dos: i)

presentan elevadas cantidades de productos de oxidación que se absorben en el intestino y contribuyen al pool de productos de oxidación que se generan in vivo y ii) estos alimentos son muy sensibles a sufrir reacciones de oxidación durante la digestión, debido a su alto contenido en hierro (carnes rojas), elevado contenido en grasas y bajo contenido en sustancias con actividad antioxidante. Como resultado, a los productos de oxidación presentes naturalmente en el alimento hay que sumarle los que se generan en el intestino como consecuencia del estrés oxidativo luminal (en el interior del tubo digestivo) (Van-Hecke et al., 2019).

Existen estudios que ponen de manifiesto los efectos perjudiciales del consumo de carne oxidada. Así, se ha descrito que el consumo de carne de cerdo oxidada produce trastornos en el metabolismo de la glucosa en ratones (Ge et al., 2020) y altera la microbiota intestinal, produciendo un aumento del peso corporal, daño en la barrera intestinal e induciendo estrés oxidativo y el desarrollo de la respuesta inflamatoria (Ge et al., 2020). Por otro lado, Van Hecke et al. (2016) concluyeron que productos cárnicos con altas cantidades de grasa eran más citotóxicos para las líneas celulares de CCR (Caco-2, HTC-116, HT-29) que aquellos con bajas cantidades de grasa. Además, el consumo de grandes cantidades de carne roja se ha asociado con mayor cantidad de proteína C reactiva en plasma (Azadbakht & Esmaillzadeh, 2009), proteína asociada a la inflamación.

Los productos de oxidación lipídica que se generan en la oxidación de la carne pueden producir alteraciones covalentes al interaccionar con moléculas de importancia biológica como ácidos nucleicos, proteínas o lípidos, originando modificaciones postraduccionales/epigenéticas que pueden provocar, a su vez, daño molecular y enfermedades (Esterbauer et al., 1993). Además, estos productos de la oxidación lipídica son capaces de difundir por las membranas celulares, contribuyendo a la alteración de la función proteica, modificando las señales celulares y produciendo daños en los tejidos (Guéraud et al., 2010).

Los α-, β- aldehídos consumidos en la dieta pueden ser absorbidos sin que sufran alteraciones tras la digestión, alcanzando la circulación sistémica (Grootveld et al., 1998). Por ejemplo, el 4 Hidroxinonenal (4-HNE) puede reaccionar fácilmente con el glutatión (GSH), proteínas o ADN, formando aductos con las cadenas laterales de los aminoácidos cisteína, histidina y lisina mediante una adición de Michael (Pizzimenti et al., 2013) o modificando la estructura de la proteína por la formación de bases de Schiff (Aldini et al., 2006). Por otro lado, la reactividad del MDA se debe a que es una molécula muy electrofílica y, por lo tanto, muy reactiva a aminoácidos básicos y a los grupos tiol (Ishii et al., 2008; Ayala et al., 2014). Se sabe que el MDA reacciona principalmente con la lisina, generando Nε- (2-propenal) lisina y produciendo enlaces cruzados de lisina-lisina con puentes de tipo 1-amino-3-

iminopropeno y piridil-dihidropiridina (Uchida, 2000) que puede dar lugar a la modificación de las respuestas celulares (Estévez et al., 2017). Además, se ha visto que el MDA tiene propiedades mutagénicas y carcinogénicas, ya que induce daños en el ADN por la formación de aductos, como el pirimido [1,2-α] purino-10(3H)-ona-2'- desoxirribosa (M1dG; aducto de ADN con MDA) (Van Hecke et al., 2017), que tiene una elevada tasa de mutagenicidad. Por los efectos que produce el MDA se considera muy relevante ampliar la investigación sobre los mecanismos moleculares de este compuesto.

Finalmente, los productos de oxidación proteica generados tras la digestión de alimentos oxidados producen daño oxidativo en las células, se acumulan y afectan a la homeostasis celular y a los procesos fisiológicos, alterando las vías de señalización celular, la microbiota intestinal o el estado redox del tejido intestinal. Además, tienen capacidad de formar aductos de macromoléculas que pueden ser la base de algunas patologías y de la modificación de la expresión génica (Estévez &Luna, 2016).

Los carbonilos que se forman tras la oxidación de proteínas se dan como resultado de ladesaminación oxidativa a la que son sometidos los aminoácidos alcalinos (lisina, prolina, arginina) por la acción de radicales libres, dando lugar a aldehídos (Estévez y Luna, 2016). Sin embargo, se ha observado que los azúcares reductores y dicarbonilos derivados de la reacción de Maillard (glioxal y metil-glioxal), interaccionan con estos aminoácidos alcalinos dando lugar a dichos aldehídos (Akagawa et al., 2005). Se ha observado que niveles incontrolados de glucosa y glioxal pueden ser responsables de la aparición de enfermedades cardiometabólicas por la inducción de estrés glicooxidativo y del daño masivo a las proteínas (Ruskovska & Bernlohr, 2015).

Como se ha mencionado anteriormente, el AAA, compuesto intermediario del metabolismo de la lisina se encuentra en cantidades considerables en productos lácteos y en carne (Soladoye et al., 2015). Este compuesto se ha identificado como un marcador de la oxidación de proteínas in vivo y de enfermedad en humanos (Lee et al., 2019). Además, en un estudio con animales de experimentación, se señala al AAA como inductor de la diabetes tras su administración oral (Wang et al., 2013). Finalmente, estudios recientes han mostrado la capacidad del AAA para alterar el estado redox y la funcionalidad de las células intestinales y pancreáticas, que confirma la implicación y la toxicidad potencial de este metabolito en el páncreas y en la regulación de la glucosa (Estaras et al., 2020; Díaz-Velasco et al., 2020). Sin embargo, las bases moleculares del mecanismo de acción del AAA sobre enfermedades no se conocen, por lo que se estima muy interesante incrementar el conocimiento en este campo.

1. 4. Estrategias antioxidantes

Dada la generación natural de radicales libres y el efecto perjudicial de los mismos, las células tienen, como ya se ha anticipado, mecanismos para hacer frente a estas sustancias y a sus efectos perjudiciales (estrés oxidativo). Entre estos mecanismos de defensa se incluyen: mecanismos preventivos, de reparación, defensas físicas y defensas antioxidantes (Betteridge, 2000).

Las defensas antioxidantes comprenden (Figura 1.3):

- Enzimas antioxidantes: SOD GPox o CAT. Previenen la formación de radicales libres y neutralizan los ya formados.
- Proteínas quelantes de metales de transición: transferrina, ceruloplasmina y albúmina cuya función es secuestrar hierro y cobre libres y evitar que participen en la reacción de Fenton.
- Antioxidantes no enzimáticos: representados por ácido ascórbico (vitamina C), α-tocoferol (Vitamina E), GSH, ácido lipoico, carotenoides o flavonoides, entre otros (Valko et al., 2007). Éstos neutralizan los radicales libres mediante la donación de electrones.

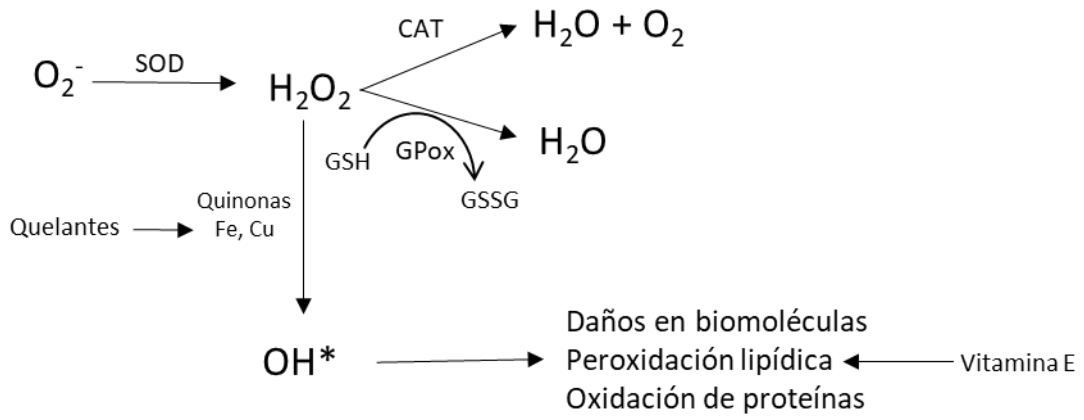

Figura 1.3: Figura resumen de las defensas antioxidantes que comprenden enzimas antioxidantes, proteínas quelantes y antioxidantes no enzimáticos.

Entre las características que los antioxidantes deben cumplir para ejercer su función de manera óptima, se encuentran: unirse específicamente a los radicales libres, secuestrar metales que tengan efecto redox, interactuar con otros antioxidantes, tener un efecto positivo sobre la expresión génica, tener fácil absorción (aquellos que tengan origen dietético), encontrarse en una concentración

fisiológicamente relevante en tejidos y biofluidos y funcionar tanto en el dominio acuoso como en el de la membrana (Valko et al., 2006).

El medio donde actúan es variable, unos actúan en un ambiente hidrofílico, como la vitamina C; otros en ambiente hidrofóbico, como la vitamina E; y algunos en ambos ambientes de la célula, como el ácido lipoico, que es soluble en agua y en grasas y, por lo tanto, puede actuar en membranas celulares y en citosol (Valko et al., 2006).

En alimentos, los efectos negativos de la oxidación de lípidos y proteínas se pueden inhibir al añadir aditivos o ingredientes con actividad antioxidante (Estévez, 2021). La oxidación que se produce en los alimentos puede ser contrarrestada, por un lado, mediante la adición de antioxidantes sintéticos (Dolores et al., 2011), hecho estrictamente regulado por los gobiernos debido a que los antioxidantes añadidos pueden tener efectos tóxicos (Shahidi & Ambigaipalan, 2015). Por ejemplo, algunos estudios en animales de laboratorio han encontrado que el hidroxibutilanisol (BHA) y el butilhidroxitolueno (BHT), dos antioxidantes fenólicos sintéticos, pueden ser responsables de daños en el hígado o carcinogénesis cuando se usan en altas concentraciones (Rodil et al., 2010; Biparva et al., 2012) y otros pueden formar complejos con la estructura de los ácidos nucleicos, causando daños en la doble hélice del ADN (Dolatabadi & Kashanian, 2010). Por ello, los antioxidantes que se utilizan en la industria alimentaria deben ser no tóxicos, baratos y efectivos en bajas concentraciones, muy estables y capaces de aguantar el procesado, no tener olor, sabor, ni color, fáciles de incorporar y tener buena solubilidad en el producto (Kiokias et al., 2008).

Debido al potencial tóxico y unido al interés de los consumidores por adquirir productos naturales, la industria considera necesario aplicar otras fuentes de antioxidantes, donde destacan los compuestos obtenidos de vegetales, como frutas, hojas o aceites esenciales (Brewer & Rojas, 2008; Estévez, 2021) de los que se extraen compuestos entre los que se encuentran los fenólicos que han demostrado ser buenas alternativas. Se pueden extraer compuestos fenólicos de residuos industriales derivados de la industria del vino o del zumo, así como de las semillas de la uva o el orujo, que mantienen su potencial antioxidante intacto (Lorenzo et al., 2018; Peixoto et al., 2018). Las propiedades antioxidantes de estos compuestos fenólicos tienen que ver con su capacidad de eliminar radicales libres, donar átomos de hidrógeno y ejercer actividad quelante sobre los cationes metálicos (Balasundram et al., 2006).

Además, los compuestos fenólicos que se añaden a los alimentos con el objetivo de controlar las reacciones de oxidación pueden tener bioactividades adicionales como efectos antimicrobianos, antiproliferativos o antiinflamatorios, entre otros (Peixoto et al., 2018; Monteiro-Espíndola et al., 2019) y, por lo tanto, tener efectos beneficiosos para la salud humana.

Una clase de compuestos fenólicos son los ácidos hidroxicinámicos, que se encuentran de forma natural en multitud de plantas y frutas (Olthof et al., 2001). En este grupo, el mayor representante es el ácido cafeico (Figura 1.4a), siendo su éster, el ácido clorogénico (Figura 1.4b) el compuesto más frecuentemente encontrado en alimentos (Scalbert & Williamson, 2000). Este compuesto está presente, principalmente, en el café y también en frutas como manzanas, peras, bayas, alcachofas y berenjenas (Clifford, 1999). El ácido clorogénico tiene efectos antiinflamatorios y antioxidantes demostrados (Liang & Kitts, 2016), así como frente a varias enfermedades como el cáncer (Liu et al., 2013), la diabetes (Meng et al., 2013) o la hipertensión (Zhao et al., 2012). Además, numerosos estudios han confirmado que el ácido clorogénico puede ser utilizado como agente antimicrobiano, que podría ser útil para la preservación de alimentos (Puupponen-Pimiä et al., 2001; Muthuswamy & Rupasinghe, 2007; Bajko et al., 2016).

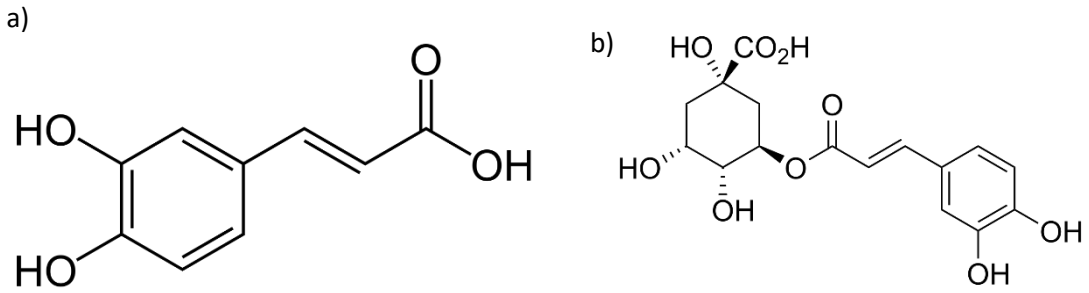

Figura 1.4: Estructura de: a) ácido cafeico y b) ácido clorogénico (Adaptado de Olthof et al., 2001)

Como alternativa a los antioxidantes sintéticos se sabe que los probióticos tienen efectos beneficiosos sobre la salud, encontrándose entre estos su potencial antioxidante, reduciendo el daño oxidativo o la tasa de radicales libres (Mishra et al., 2015). Los probióticos son microorganismos vivos que, al ser ingeridos en cantidad adecuada, producen un efecto favorable en el equilibrio de la población microbiana intestinal, al potenciar el sistema inmunológico (Guarner et al., 2010). Los microorganismos probióticos facilitan la digestión al acidificar el tubo digestivo, por la producción de ácido láctico, gracias a lo que también se frena el crecimiento de otras bacterias intestinales indeseables que producen toxinas o intervienen en el proceso de putrefacción.

Otros efectos beneficiosos que se han atribuido a los microorganismos probióticos son la mejora de la población microbiana, la estimulación de la mucosa intestinal, la reducción de las reacciones inflamatorias o alérgicas y de los niveles de colesterol sanguíneo (Mercenier et al., 2003; Reid et al., 2003; Tuohy et al., 2003), así como su capacidad de influir en el estado oxidativo, teniendo efecto antioxidante en la mucosa intestinal (Kullisaar et al., 2012). También hay que tener en cuenta que la

presencia de probióticos en productos alimenticios no debe generar impactos negativos en la calidad, ni afectar a sus características sensoriales (Stanton et al., 2003).

Sin embargo, para el uso de probióticos en la alimentación humana no existe una legislación específica para su regulación, por lo que tampoco existen requisitos específicos para los mismos, ni una lista de productos autorizados, aunque esto no quiere decir que no se puedan emplear en la alimentación. En ausencia de una lista de microorganismos autorizados, a nivel de la Unión Europea, se toma como referencia la lista QPS (*Qualified presumption of safety*) de la Autoridad Europea de Seguridad Alimentaria (EFSA), que se revisa periódicamente (EFSA BIOHAZ Panel, 2021).

El uso de probióticos en alimentación está sujeto a los requisitos generales establecidos en el Reglamento (CE) nº 178/2002 del Parlamento Europeo y del Consejo, de 28 de enero de 2002*, por el que se establecen los principios y los requisitos generales de la legislación alimentaria, se crea la Autoridad Europea de Seguridad Alimentaria y se fijan procedimientos relativos a la seguridad alimentaria*. En particular, para el empleo de estos probióticos debe tenerse en cuenta que el artículo 14 del Reglamento que establece, entre otras condiciones, que no se comercializarán los alimentos que no sean seguros.

Las características a tener en cuenta para la selección de microorganismos probióticos tienen que ver con aspectos tecnológicos, de seguridad y funcionalidad, como la tolerancia a las condiciones del estómago y del intestino delgado, la adherencia a la mucosa intestinal y la persistencia en el tracto intestinal (Pidcock et al., 2002; Pennacchia et al., 2004; Begley et al., 2005).

En la industria agroalimentaria se utiliza una amplia variedad de microorganismos (Tabla 1.1), por su carácter probiótico, perteneciendo en su mayoría al grupo de bacterias ácido-lácticas (BAL), así como a los géneros *Bacillus, Propionibacterium*, levaduras y mohos filamentosos (Ruiz-Moyano et al., 2008).

Tabla 1.1. Microorganismos más comúnmente utilizados como probióticos (Fuente: Iñiguez et al., 2014).

Bacterias ácido-lácticas (BAL)			**Bacterias no ácido-lácticas y levaduras**
***Lactobacillus* spp.**	***Bifidobacterium* spp.**	**Otras especies de BAL**	
L. acidophilus *L. amylovorus* *L. casei* *L. crispatus* *L. delbrueckii subsp. bulgaricus* *L. gallinarum* *L. fermentum* *L. gasseri* *L. johnsonii* *L. paracasei* *L. plantarum* *L. reuteri* *L. rhamnosus*	*B. adolescenctis* *B. animalis* *B. bifidum* *B. breve* *B. longum* *B. lactis* *B. infantis*	*Enterococcus faecalis* *Enterococcus faecium* *Lactococcus lactis* *Leuconostoc mesenteroides* *Pediococcus acidolactici* *Streptococcus oralis* *Streptococcus uberis* *Streptococcus rattus*	*Bacillus cereus* var. *toyoi* *Escherichia coli* *Propionibacterium fredeunreichii* *Sporolactobacillus inulinus* *Saccharomyces cerevisias* *Saccharomyces boulardii*

Las BAL constituyen un grupo de bacterias ampliamente distibuidas, donde están presentes diferentes géneros relacionados filogenéticamente, que tienen características bioquímicas, fisiológicas y ecológicas en común (Adams, 1999). Las funciones de las BAL están relacionadas con sus características como probióticos, se añaden a alimentos por sus propiedades beneficiosas, siendo las más utilizadas (Zielinska & Kolozyn-Krajewska, 2018) o bien se administran como suplementos alimenticios (Hirayama & Rafter, 1999). Por otro lado, respecto a sus características tecnológicas, poseen gran importancia en la industria alimentaria ya que se emplean como cultivos iniciadores para elaborar productos fermentados, como los derivados de la leche (Evivie et al 2017) o de la carne (Ruiz-Moyano et al 2008).

Muchas de estas bacterias que se utilizan como probióticos han sido aisladas del intestino, o de heces humanas (*L. salivarius* subsp. s*alicinius*, *L. acidophilus, B. longum*) y, con menor frecuencia, del estómago (*L. fermentum* o *L. salivarius*) (Zielinska & Kolozyn-Krajewska, 2018) ya que naturalemente colonizan el tracto gastrointestinal. Sin embargo, una gran cantidad de cepas probióticas pueden ser

aisladas de productos fermentados, siendo los productos tradicionales una fuente importante de microorganismos con estas propiedades (Zielinska & Kolozyn-Krajewska, 2018).

La función principal como probióticos es reforzar el sistema inmune humano cuando se administran en la dieta en cantidades adecuadas. Exsiten estudios en los que se ha señalado que se pueden prevenir enfermedades mediante la administración de BAL, por ejemplo, como defensa contra el desarrollo de alergias (Cross & Gill, 2001), prevención y tratamiento del CCR (Kahouli et al., 2013), contra diabetes y obesidad, minimizando el riesgo asociado a esta enfermedad (Andreasen et al., 2010), prevención y tratamiento del síndrome de intestino irritable (Guerra et al., 2011), entre otras aplicaciones frente a enfermedades. En concreto, frente a las enfermedades inflamatorias intestinales se ha señalado que la administración de BAL como tratamiento ayuda a restaurar la microbiota intestinal dañada en estas patologías (Ayivi et al., 2020).

Entre las aplicaciones en la industria alimentaria, las BAL han sido utilizadas como cultivos iniciadores en una gran variedad de productos. Un cultivo iniciador puede ser definido como "una preparación microbiana de un gran número de células de al menos un microorganismo que se añade a una materia prima para producir un alimento fermentado acelerando y dirigiendo este proceso" (Leroy & De Vuyst, 2004). En este sentido, las BAL confieren al producto unas características relevantes para su procesado como, por ejemplo, produciendo una rápida acidificación de la materia prima mediante la producción de ácido láctico; también generan ácido acético, etanol, bacteriocinas u otras enzimas, todo ello lleva a aumentar la vida útil, mejorar la seguridad alimentaria, mejorar la textura y contribuir a las características sensoriales del producto final (Leroy & De Vuyst, 2004).

En la industria cárnica las bacterias ácido-lácticas han sido ampliamente utilizadas. Principalmente como cultivos iniciadores, en la elaboración de derivados cárnicos curado-madurados, para obtener productos seguros y estables desde el punto de vista microbiológico, tecnológico y económico, aportando, como ya se ha señalado previamente, beneficios probióticos (Ammor y Mayo, 2007; Arihara, 2006).

Uno de los probióticos ampliamente utilizado como suplemento alimenticio para mejorar la salud del intestino humano es *Lactobacillus reuteri*, colonizador natural del tracto gastrointestinal en humanos y animales (Shornikova et al., 1997). La administración oral de *L. reuteri* reduce las enfermedades e infecciones y contribuye a una microflora del colon bien balanceada (Shornikova et al., 1997). En relación con los mecanismos detrás de su efecto probiótico, se ha visto que *L. reuteri* protege frente al estrés oxidativo e inhibe el aumento de productos de oxidación en el lumen (Amaretti et al., 2013). Mientras los beneficios de *L. reuteri* contra el estrés oxidativo y enfermedades del intestino están

documentados (Petrella, 2016) los mecanismos moleculares implicados en las respuestas de estas bacterias probióticas bajo condiciones prooxidantes (como las inducidas por MDA u otros productos de oxidación lipídica o proteica) no están bien definidos. A este respecto, en un estudio previo de Arcanjo et al., 2019 hemos observado, de manera preliminar, la base genética y molecular de las respuestas de *L. reuteri* contra el estrés oxidativo inducido por H_2O_2, sin embargo, son necesarios nuevos estudios para aclarar completamente los mecanismos moleculares de las bacterias frente a estrés oxidativo.

Otro microorganismo que tiene capacidades probióticas es *Enterococcus faecium*, que demostró actuar de manera beneficiosa frente a la diarrea asociada a antibióticos (Gismondo et al., 1999), así como sobre los factores cardiovasculares, reduciendo la concentración de colesterol (Hlivak et al., 2005). Además, ciertas cepas de *E. faecium*, aisladas de productos cárnicos curados, tienen capacidad de producir bacteriocinas frente a *Listeria monocytogenes*, que sugiere que estas cepas se puedan utilizar como cultivos iniciadores adjuntos para mejorar la seguridad alimentaria (Casaus et al., 1997; Cintas et al., 2000).

El potencial de las BAL para proteger frente a condiciones oxidantes producidas en las enfermedades inflamatorias intestinales es bien conocido. Sin embargo, los mecanismos moleculares mediante los que se lleva a cabo esta acción son desconocidos, por ello es importante dilucidar las rutas implicadas en esta respuesta, mediante la aplicación de técnicas avanzadas que ayuden a este fin.

1. 5. Técnicas de análisis molecular avanzado para su aplicación al estudio de las respuestas de microorganismos frente a estrés oxidativo

Para estudiar las interacciones moleculares que se producen entre los productos de oxidación de la dieta y las bacterias probióticas se requieren métodos avanzados de estudio.

El estudio de la oxidación proteica es esencial para entender las bases moleculares de enfermedades en las que el estrés oxidativo juega un papel principal. A este respecto, hay poca información disponible en cuanto a las respuestas biológicas de las bacterias probióticas a especies oxidantes. Para entender cómo influyen factores externos en las particularidades biológicas de las bacterias y de sus rutas metabólicas, el análisis de la expresión génica, del proteoma y de las características celulares son herramientas valiosas y altamente específicas.

1.5.1 Expresión génica aplicada al estudio de respuestas de microorganismos

La expresión génica es el proceso mediante el cual la información codificada en los ácidos nucleicos se transforma en proteínas. Esta síntesis de proteínas tiene lugar en dos pasos: transcripción y traducción. En la transcripción una de las hebras que conforman la doble hélice de ADN sirve como molde para sintetizar una molécula de ARN de cadena sencilla. Posteriormente, esta hebra de ARN llega a los ribosomas, donde se genera la cadena de aminoácidos y, por tanto, la proteína. Esta técnica, se basa en conocer como la información de un gen se está utilizando en la síntesis de un producto génico funcional, fundamental para la diferenciación celular, la morfogénesis y la adaptación del organismo a su entorno (Mazza & Mazzette, 2014). Con este análisis es posible evaluar los genes implicados en la adaptación de las BAL a un entorno concreto o cuál es su respuesta ante un estímulo determinado, como las condiciones de estrés oxidativo generadas por productos oxidantes resultantes de la digestión de lípidos y proteínas.

Para el estudio de la expresión génica la técnica elegida, mayoritariamente, es la PCR en tiempo real o PCR cuantitativa (qPCR), con la que se obtienen datos precisos y reproducibles de determinados genes seleccionados. Para ello, se utiliza el ARN extraído de las muestras de interés, que se transcribe a ADN complementario (cDNA) mediante la transcripción inversa (RT-PCR) y este es utilizado para determinar la expresión relativa de genes diana frente a genes control.

A diferencia de la PCR convencional donde el producto de PCR se detecta mediante la separación electroforética en un gel de agarosa al finalizar la reacción, en la qPCR el producto amplificado es detectado y medido mientras avanza la reacción. La detección del producto de PCR es posible al incluir en la reacción una molécula fluorescente que informa del incremento de la cantidad de ADN amplificado que es proporcional a la señal fluorescente. Es necesario utilizar termocicladores especializados equipados con módulos de detección de fluorescencia que registran la cantidad de producto amplificado en cada ciclo, permitiendo determinar, con una alta precisión, el número de copias de ADN tanto en el inicio de la reacción como en cada uno de los ciclos sucesivos. Los resultados de la qPCR pueden ser cualitativos (indicando presencia o ausencia) o cuantitativos (determinando la cantidad de ácido nucleico presente en una muestra), en cuyo caso se conoce como PCR cuantitativa o qPCR (Real-Time PCR Applications Guide, 2006, Bio-Rad Laboratories, Inc).

La amplificación es registrada como se muestra en la Figura 1.5, donde el eje de abcisas (X) muestra el número de ciclo de PCR y el eje de ordenadas (Y) la fluorescencia acumulada de la amplificación, que es proporcional a la cantidad del producto amplificado. Como se observa en la gráfica, existen dos fases, una exponencial, en la que la cantidad de producto de PCR se duplica, aproximadamente, en

cada ciclo; y otra de *plateau* (estacionaria) al final de la reacción en la cual los componentes se han cosumido. Cuando hay suficiente cantidad de producto amplificado, la fluorescencia es detectable, denominándose el número de ciclo en el que se produce este hecho *thresold cycle* o ciclo umbral (C_T). El valor de C_T dependerá de la cantidad inicial de ADN.

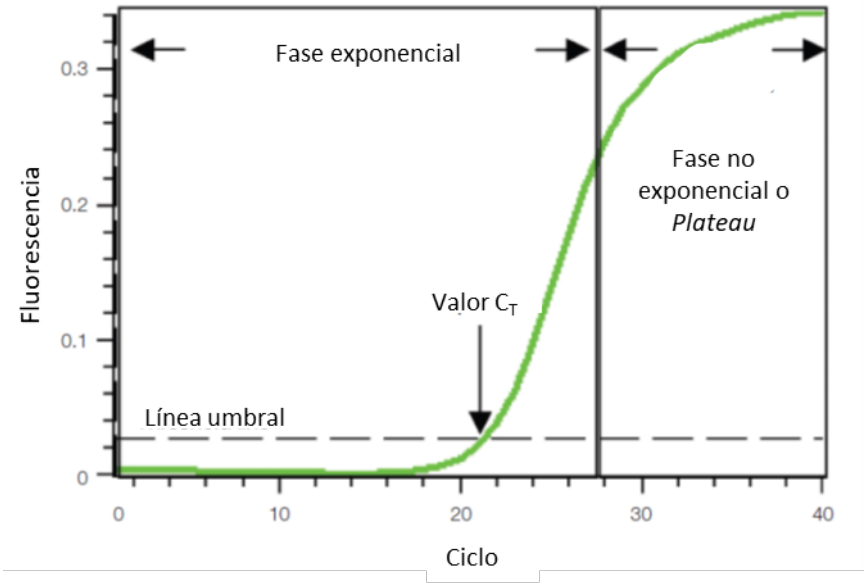

Figura 1.5: Gráfico de amplificación de una qPCR, donde se muestra la fase exponencial y el ciclo umbral (C_T value) de la reacción (Fuente: Bio-Rad Laboratories, Inc, 2006).

Como ya se ha señalado, la qPCR es utilizada para determinar la cantidad de ácidos nucleicos en una muestra biológica. Existen dos métos de análisis para llevar a cabo esta cuantificación: absoluta y relativa.

- Cuantificación absoluta: compara los valores de C_T de las muestras con una curva estándar de concentraciones conocidas de ADN, realizada previamente.
- Cuantificación relativa: se analizan los cambios de expresión génica de un gen en estudio o diana en relación con una muestra de referencia sin tratar (grupo control). Por ejemplo, se puede evaluar la variación de la expresión de un gen en un momento y con un tratamiento determinado frente a muestras sin tratar. Los resultados se expresan como número de veces que aumenta o disminuye la expresión génica frente al control.

Como en esta Tesis Doctoral se ha empleado la cuantificación relativa, a continuación, se desarrollarán las características fundamentales de la misma. Para cuantificar los cambios relativos en la expresión génica utilizando qPCR se requiere un buen análisis de los datos. Para ello, el método de $2^{-\Delta\Delta Ct}$ descrito por Livak & Schmittgen (2001) destaca la importancia de un correcto proceso de normalización, que

evite el error derivado del experimento y que garantice la validez de los resultados y su reproducibilidad. Para llevar a cabo la normalización, los factores a tener en cuenta son:

- Control endógeno o gen de referencia que normalice la expresión de los genes diana, cuya expresión debe ser constante entre experimentos. Se utilizan genes *housekeeping* y, frecuentemente en bacterias, el gen de la subunidasd 16S del ARNr como control endógeno por su alto grado de conservación y expresión constante.
- Calibrador o muestra sin tratamiento, empleada como muestra control.
- Cantidad y calidad del ARN total empleado que puede influir en la cuantificación final de la expresión génica (Huggett et al., 2005).
- La eficiencia en la síntesís de ADNc depende de la calidad del ARN extraído y de una buena reproducibilidad de la RT utilizada. El ARN se retrotranscribe a ADNc que es finalmente utilizado para la qPCR.
- Y la eficiciencia de los cebadores de los genes en estudio, que depende del diseño de los mismos y del tipo de fluorescencia empleada. Su eficiencia de amplificación debe ser evaluada mediante una curva estándar.

En el diseño del método de qPCR es importante la elección del tipo de fluorescencia utilizada para monitorizar la amplificación de la secuencia diana. Se pueden diferenciar dos tipos: fluorescentes que se unen al ADN (SYBR Green I) y sondas o cebadores específicos de secuencia marcados con fluorescencia (las más utilizadas son las sondas TaqMan).

Entre los fluorescentes que se unen al ADN, el más común es el SYBR Green I, que se asocia de manera no específica a la doble cadena de ADN (dsDNA). Este fluorocromo tiene poca fluorescencia cuando está libre en solución, pero cuando se une a dsDNA aumenta hasta mil veces. Por lo tanto, el total de fluorescencia de una reacción es proporcional a la cantidad de dsDNA presente e incrementará si la diana se amplifica (Bio-Rad Laboratories, Inc, 2006).

Las sondas TaqMan (Figura 1.6) son específicas de secuencia y se marcan con fluorescencia. Se basan en la actividad 5’ exonucleasa de ciertas polimerasas. La sonda tiene un “indicador” fluorescente en el extremo 5’ y un “extintor” en el extremo 3’. Cuando está intacto, la fluorescencia del “indicador” se apaga por su proximidad con el “extintor”. En la hibridación-extensión, la sonda se une a la diana y la actividad exonucleasa 5’ --> 3’ específica de la polimerasa escinde al “indicador”. Como resultado, el “indicador” se separa del “extintor” produciendo una señal de fluorescencia proporcional a la cantidad de producto amplificado en la muestra.

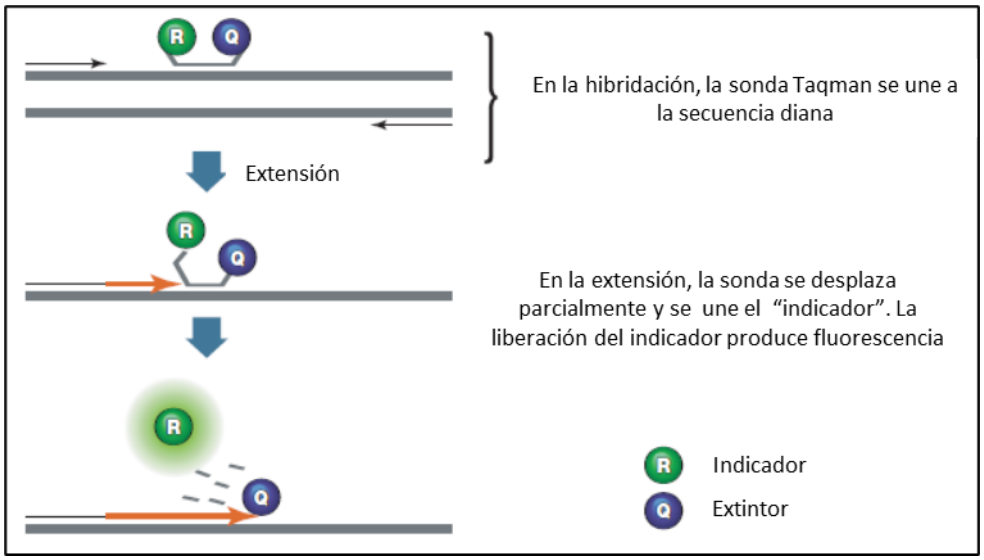

Figura 1.6: Mecanismo de acción de las Sondas TaqMan (Fuente: Bio-Rad Laboratories, Inc, 2006).

Esta técnica se utiliza de manera rutinaria en diferentes campos científicos. Concretamente en el ámbito de la microbiología existen numerosos ejemplos como la identificación de contaminación bacteriana en muestras sanguíneas (Dreier et al., 2007); la detección de bacterias patógenas en alimentos, con objeto de asegurar la higiene y seguridad alimentaria (Hanna et al 2005); la evaluación de la capacidad de las BAL para inhibir la reacción inflamatoria producida por agentes infecciosos (Tsai et al., 2018); o la cuantificación de bacterias en muestras del mar Báltico Central (Labrenz et al., 2004).

La qPCR se utiliza también para determinar la infección por virus en animales, humanos o alimentos, por ejemplo, se han detectado norovirus, que produce gastroenteritis, en quesos o lechuga (Fumian et al., 2009); o el virus de la Hepatitis A en salsa de tomate y fresas (Love et al., 2008). Recientemente, se emplea esta técnica para detectar las infecciones por el SARS-Cov2, pudiendo determinar la cantidad de copias presentes en el hospedador (Jawerth, 2020).

Como se ha comentado previamente, el análisis de la expresión génica es una herramienta valiosa y altamente específica para comprender la influencia de los factores externos sobre determinadas rutas y funciones metabólicas de las bacterias, mediante el estudio de la regulación de determinados genes relacionados con las condiciones ambientales. Uno de los primeros estudios llevados a cabo con esta técnica está relacionado con la respuesta *in vitro* de *Staphylococcus epidermis* a varios tipos de estrés (Vandecasteele et al., 2001). Además, Beltramo et. al (2006) cuantificaron la expresión de varios genes de *Oenococcus oeni* en respuesta a condiciones de estrés acídico en la fermentación maloláctica del vino y la qPCR se ha aplicado en la determinación de los efectos de diferentes combinaciones de

antibióticos sobre el total de bacterias activas (Aydin et al., 2015) o en la evaluación de la expresión de genes de virulencia de *Listeria monocytogenes* en jamón curado (Alía et al., 2019) entre otros trabajos.

Los genes implicados en la respuesta de las BAL a estrés son numerosos; sin embargo, el nivel de caracterización de su función y regulación de cada uno de ellos es diferente entre las especies que conforman el grupo (Van de Guchte et al 2002). La identificación de genes específicos de respuesta a estrés puede dar información sobre la manipulación de las bacterias, para potenciar o inhibir el crecimiento, desarrollar herramientas para identificar cepas tolerantes o sensibles y para evaluar el nivel de adaptación de un cultivo (van de Guchte et al 2002).

En este sentido, mediante qPCR se detectó la sobreexpresión de genes que codifican para respuestas redox en *Bifidobacterium animalis* subsp. l*actis* bajo condiciones de estrés oxidativo (Feng & Wang, 2020). De forma similar, se ha descrito la activación de genes como el *uspA* o el *dhaT* en *L. reuteri* en respuesta al estrés oxidativo causado por ROS (Arcanjo et al., 2019). La superfamilia de proteínas de estrés universal A (*universal stress protein A*, uspA) es un grupo de proteínas muy conservadas de microorganismos, insectos y plantas (Kvint et al., 2003). La función específica de las proteínas Usp en los sistemas biológicos todavía no es conocida, aunque parece estar implicada en la defensa contra agentes que dañan el ADN (Kvint et al., 2003). Por su parte, se ha descrito que el gen *dhaT* está implicado en la síntesis de 1,3 propanodiol oxidorreductasa (1,3-PDO), que interviene en convertir la reuterina (compuesto antimicrobiano) en 1,3 propanodiol, pudiendo llevar a una sobreproducción de la primera (Schaefer et al., 2010). Sin embargo, la conexión entre el estrés oxidativo, la expresión génica y la oxidación de proteínas en bacterias probióticas todavía no se ha descifrado.

Además, en algunos estudios, las herramientas genómicas avanzadas han sido cruciales para revelar las bases moleculares de los efectos beneficiosos de *L. reuteri* frente a bacterias patógenasa como *E. coli* enterohemorrágica O157:H7 (Carey et al., 2008) y el efecto antiproliferativo de *L. reuteri* en las células humanas de leucemia mieloide (Iyer et al., 2008).

1.5.2. Proteómica en el estudio de las respuestas de BAL a estrés

La proteómica es un área de la Biología Molecular que tiene como objetivo estudiar el conjunto completo de proteínas expresadas por un genoma, una célula o un tejido (identificación, cuantificación y localización), así como las interacciones que se producen entre ellas y las funciones que tienen en el organismo (Anderson & Anderson, 1998).

En el estudio de la proteómica, se consideran claves las modificaciones postraduccionales para definir sus funciones, en las que se incluyen alteraciones covalentes que preparan a la proteína para su

función, produciendo el plegamiento y dirigiéndola a su destino específico. Las alteraciones covalentes comprenden el procesamiento proteolítico, la modificación de las cadenas laterales de los aminoácidos y la inserción de cofactores (Torrades, 2004).

Las aplicaciones de la investigación proteómica son muchas y variadas (Torrades, 2004) ya que el análisis a gran escala del proteoma aporta conocimiento entre otros campos en investigación básica, para la mejora del conocimiento de los organismos; en biomedicina, al descifrar el mecanismo de aparición de enfermedades; o en farmacia, obteniendo información de proteínas diana para fármacos, así como para el diseño de los propios fármacos (Domon & Aebersold, 2006). Además, este análisis permite identificar proteínas presentes en muy baja concentración.

En ciencia de los alimentos, la proteómica permite desarrollar métodos para su aplicación de manera rutinaria en el análisis de alimentos y, entre otras muchas aplicaciones en este campo, se encuentran la validación y optimización de procesos y control de calidad de los mismos, la identificación de microorganismos patógenos, proteínas alergénicas o compuestos bioactivos de interés o la caracterización de materias primas y detección de variaciones entre lotes (Han & Wang, 2008, Gašo-Sokač et al., 2010).

En el estudio del proteoma se han desarrollado diversos métodos, que se pueden diferenciar entre los basados en electroforesis en geles de acrilamida, donde se encuentra uno de los primeros métodos desarrollados como es la electroforesis en gel bidimensional (2-DE), mediante la que se realiza la separación de proteínas en dos dimensiones: la primera según su punto isoeléctrico y la segunda según su peso molecular (Ranjbar et al., 2017). Otros métodos disponibles son los no basados en gel como la separación mediante cromatografía líquida de alto rendimiento (HPLC), con la posterior identificación por espectrometría de masas (MS) que permite separar e identificar mezclas de péptidos complejas (Koskenniemi, 2012).

Uno de los métodos en los que se emplea la MS es la proteómica de abajo hacia arriba o “botton-up” que permite el estudio de las estructuras de las proteínas y da información de todo el proteoma de la célula en su entorno, pudiendo dilucidar las respuestas de las células en unas condiciones determinadas (Yuan et al., 2019).

Independientemente de la técnica empleada para el estudio del proteoma, es necesario realizar la extracción y purificación de las proteínas de la muestra para lo cual la principal dificultad es la lisis celular, paso considerado limitante, y la calidad del análisis depende de su eficiencia (Wisniewski, 2018). A continuación, una de las estrategias llevadas a cabo para seguir con el análisis, es la digestión

de las proteínas mediante enzimas como la tripsina, que es la más comúnmente utilizada. Los péptidos que se obtienen de esta digestión son detectados por el espectrómetro de masas. El análisis bioinformático final permite identificar y cuantificar las proteínas comparando los resultados con bases de datos.

Son numerosos los estudios en los que se ha utilizado la proteómica como herramienta para caracterizar la funcionalidad de bacterias y otros microorganismos, así como su respuesta y adaptación a factores ambientales relevantes (Ruiz et al., 2016). Entre los estudios desarrollados mediante esta tecnología se encuentran: la aplicación de técnicas ómicas integradas con MS para entender los mecanismos que desencadenan la virulencia de las bacterias (Man et al., 2021); por su parte, Mbye et al., (2020) recogieron, en una revisión, la respuesta de diversas bacterias probióticas a distintos tipos de estrés (por calor, frío, acídico u oxidativo) señalando las proteínas que se inducían en cada caso. Además, se han estudiado los cambios producidos en el proteoma de *L. acidophilus* como respuesta al cultivo de este probiótico con compuestos fenólicos de plantas (Celebioglu et al., 2018) o la tolerancia de *Pediococcus pentosaceus* aislado de alimentos a H_2O_2 (Zhang et al., 2021). En estudios llevados a cabo en otros microorganismos, se ha evaluado, mediante proteómica no dirigida, el mecanismo de acción de agentes de biocontrol frente a *Penicillium nordicum* productor de ocratoxina A (Álvarez et al., 2021)

1.5.3. Citometría de flujo

La citometría de flujo es una técnica biofísica de análisis celular que se basa en tecnología láser. Mediante este método se pueden analizar y cuantificar de manera simultánea múltiples características celulares mientras son transportadas por un fluido y cuando les incide un haz de luz. El citómetro de flujo mide el tamaño, la morfología y la fluorescencia relativa de las células. Además, se utiliza para analizar la expresión de la superficie celular y las moléculas intracelulares, caracterizar y definir diferentes tipos de células en una población celular heterogénea, evaluar la pureza de subpoblaciones aisladas y analizar el tamaño y el volumen de las células. Permite un análisis multiparamétrico simultáneo de células individuales (Pérez-Lara et al., 2018).

Estas características se determinan usando un sistema óptico acoplado a un procedimiento electrónico que graba la manera en que la célula dispersa el haz de luz y emite fluorescencia (Marti et al., 2001).

Para la determinación de las características celulares, el citómetro de flujo está compuesto por tres sistemas principales:

- Sistema de fluidos, su principal función es alinear y transportar a las células dentro de la cámara de flujo hacia el haz de luz, por tanto, es necesario que la muestra esté en un fluido (Hoffman, 2008).
- Sistema óptico, está compuesto por láseres y filtros que se encargan de iluminar a las células y dirigir las señales resultantes hacia los detectores. Con este sistema se puede conocer el tamaño y la morfología celular, así como las proteínas que se expresan (marcadores), permitiendo la identificación de los tipos celulares.
- Sistema electrónico, traduce la señal luminosa generada por el haz de luz que incide en la célula en señales electrónicas. Consta de sensores luminosos como forodiodos y fotomultiplicadores, que convierten los fotones en electrones y éstos en corriente eléctrica, que se traduce a gráficos e histogramas (Marti et al., 2001)

Un paso muy importante para el análisis por citometría de flujo es el marcaje con anticuerpos monoclonales acoplados a fluorocromos, que permiten detectar y etiquetar poblaciones específicas de células. Los anticuerpos monoclonales se crean para que sean capaces de unirse a una estructura específica (antígeno) que se expresa en el tipo celular que se quiere identificar. Este anticuerpo debe tener unido covalentemente un fluorocromo que emitirá la luz fluorescente cuando sea excitado por el láser (Mao & Mullins, 2010). Hay distintos tipos de fluorocromos que emiten fluorescencia a distintas longitudes de onda, esto permite estudiar distintas poblaciones celulares a la vez.

El análisis de los resultados obtenidos se realiza mediante la representación en gráficos de puntos, histogramas, figuras tridimensionales, etc. Donde se representan la relación entre los marcadores, la frecuencia relativa, la intensidad de expresión de algún marcador o comparaciones entre varias poblaciones. Lo importante es seleccionar los gráficos que reflejen los resultados con precisión y sin generar confusiones.

Con la citometría de flujo se pueden analizar funciones celulares como la proliferación, la fagocitosis y la apoptosis o identificar, caracterizar y separar poblaciones celulares (Mattanovich & Borth, 2006). En bacterias esta técnica ha sido utilizada empleando la sonda CellROx Green en *E. coli*, *Mycobacerium smegmatis* y *M. bovis* detectándose la generación desuperóxido, señalando cómo la utilización de este método proporciona información precisa de la cantidad de ROS en bacterias estresadas (McBee et al., 2017). Para medir el estrés oxidativo, se han utilizado también combinaciones de sondas como CellROx Deep Red y SYTOX Blue (Manoil & Bouillaguet, 2018). Recientemente, Fallico et al. (2020) desarrollaron un protocolo multiparamétrico de citometría de flujo en *L. rhamnosus GG* con el que evaluaban la cantidad total de ROS libres y la integridad de membrana. Por lo tanto, con este método

avanzado es posible evaluar las respuestas de las bacterias probióticas frente a condiciones de estrés oxidativo, evaluándose cómo afectan las ROS a la integridad de membrana o al ADN bacteriano.

2. PLANTEAMIENTO Y OBJETIVOS

2. PLANTEAMIENTO Y OBJETIVOS

Teniendo en cuenta i) la importancia de los productos de oxidación de lípidos y proteínas en la inducción de efectos nocivos e incluso enfermedad en humanos, ii) el interés en aplicar estrategias dietéticas antioxidantes basadas en bacterias probióticas y fitoquímicos y iii) la necesidad de conocer las respuestas de bacterias probióticas en condiciones de estrés oxidativo, el **principal objetivo** de esta Tesis Doctoral es identificar los mecanismos moleculares implicados en la respuesta de bacterias probióticas frente a la amenaza del estrés oxidativo mediante el empleo de métodos avanzados de análisis.

Para cumplir con este objetivo general se plantearon los siguientes objetivos parciales:

- Estudiar el efecto de la exposición a MDA sobre las respuestas moleculares de una bacteria probiótica.

- Identificar los mecanismos a través de los cuales el MDA es capaz de inducir la oxidación de proteínas bacterianas.

- Entender los posibles mecanismos implicados en la detoxificación del MDA por parte de bacterias probióticas.

- Conocer los efectos potencialmente nocivos de la exposición al AAA por parte de bacterias probióticas y dilucidar los mecanismos bacterianos para hacer frente a dicha amenaza.

- Profundizar en el conocimiento de los efectos sinérgicos de compuestos fenólicos y bacterias probióticas en condiciones de estrés oxidativo generado por radicales libres

3. MATERIAL Y MÉTODOS

3.1 Material

3.1.1. Reactivos químicos

Todos los compuestos químicos y reactivos utilizados en esta Tesis Doctoral fueron del grado analítico ACS (American Chemical Society) y adquiridos en las casas comerciales Sigma-Aldrich (Darmstadt, Alemania), Scharlab S.L. (Barcelona, España), Condalab (Madrid, España), Applied Biosystems (Whaltham, MA, EE. UU.), Epicentre (Madison, WI, EE. UU.) y Acros Organics (Madrid, España).

Para la extracción de ADN y ARN de las bacterias se utilizó el kit comercial Master PureTM Complete DNA and RNA Purification Kit de la casa comercial Epicentre.

En el desarrollo de las técnicas moleculares, los cebadores fueron suministrados por la casa comercial Metabion International AG (Planegg, Alemania). Para las reacciones de transcripción inversa se utilizó el kit Prime ScriptTM RT Reagent (Perfect Real Time) y para las reacciones de qPCR SYBR® Premix Ex TaqTM II, ambos de la casa comercial Takara Bio Inc (Kusatsu, Japón).

En el análisis proteómico se utilizó tripsina de grado de secuenciación (Promega, Madison, WI, EE. UU.) y tensioactivo ProteaseMAX (Promega).

Las sondas de citometría de flujo fueron CellROX™ Deep Red Reagent de ThermoFisher (Whaltham, MA, EE. UU.) y Hoechst 33342 de Sigma Aldrich (Alemania).

3.1.2. Medios de cultivo

Las BAL se incubaron en el medio de cultivo MRS (De Man, Rogosa, Sharpe; Condalab), siguiendo las instrucciones del fabricante: (52,25 g de MRS en 1L de agua destilada), para los medios líquidos y añadiendo 12 g de agar bacteriológico para los medios sólidos.

Para llevar a cabo el recuento, los cultivos de bacterias fueron diluidos en agua de peptona al 1% (p/v). Para la conservación de los cultivos se utilizó glicerol al 20% (v/v) (Fisher Scientific, EE. UU).

Los agentes oxidantes utilizados para el cultivo de las bacterias fueron Ácido D-2-aminoadipico 98% (AAA) y Malonaldehído bis (dietil acetal) 97% (MDA), ambos de la casa comercial Acros Organics (España). El peróxido de hidrógeno (H_2O_2) pertenecía a la casa comercial Sigma-Aldrich y el ácido clorogénico a la casa comercial Sigma-Aldrich.

En el ensayo con *E. faecium*, se utilizó Dimetil sulfóxido (DMSO) 99,5% de la casa comercial Sigma-Aldrich. Los cultivos de esta bacteria se hicieron en jarras de anaerobiosis, utilizando los sobres Anaerogen™ 3,5 L de Thermo Scientific.

3.1.3. Material biológico

En esta Tesis Ddoctoral se han utilizado dos cepas de bacterias ácido-lácticas que pertenecen a la colección de cultivos del grupo de investigación de Calidad y Microbiología de los Alimentos de la Universidad de Extremadura. Las cepas en cuestión fueron: *L. reuteri* PL503 aislado de heces de cerdo, identificado por Ruiz-Moyano et al., (2008) y *E. faecium* Q233 aislado de queso tierno curado, identificado por Ordiales et al., (2013).

En todos los casos los cultivos stock se almacenaron a -80 °C en MRS líquido suplementado con glicerol en una concentración final de 20% (v/v) y previamente a su uso fueron cultivadas dos veces: *L. reuteri* en condiciones de microaerofilia *y E. faecium* en anaerobiosis a 37 °C durante 24 h, en MRS suplementado con 0,05% de ácido acético 10% (v/v) y en MRS suplementado con L-Cisteína, respectivamente.

3.1.4. Equipos

El agua destilada y ultrapura utilizadas para la elaboración de medios de cultivo, tampones, resuspensión de reactivos, etc., han sido obtenidas mediante el sistema de purificación de agua Elix Technology Inside Milli-Q® Integral 5 Water System de Merck Millipore (Burlington, MA, EE. UU.).

Las pesadas rutinarias se realizaron en una balanza electrónica CB Complet de Cobos, con una precisión de 10 mg. Para pesadas de precisión se utilizó una balanza analítica mod. LA310S, de Sartorius, con una precisión de 0,1 mg.

Los medios de cultivo y los tampones se prepararon en agitadores magnéticos con placa calefactora mod. Agimatic-N de JP Selecta.

La esterilización de medios de cultivo, reactivos, tampones y material de laboratorio se realizó en autoclave mod. Presoclave III 80 de P-Selecta. Para atemperar los medios de cultivo y llevar a cabo ciertos pasos de los protocolos de laboratorio que requerían temperatura controlada se utilizaron baños termorregulados mod. Prescidig de P-Selecta. Las placas Petri utilizadas para los medios de cultivo, de 90 mm de diámetro, de la casa comercial Fisher Scientific. La conservación de los medios de cultivo se hizo a 5 °C en una cámara refrigerada por un equipo de frío RivaCold mod. RC325-45ED.

Para el almacenamiento de las muestras a temperatura de -20 °C se utilizaron congeladores de las marcas Lynx y Liebherr MedLine.

Las mediciones de pH se realizaron con un pHmetro Crison mod. BASIC20.

Las manipulaciones que requerían condiciones de esterilidad fueron realizadas en una campana de flujo laminar TELSTAR mod. BV-100 equipada con lámpara UV.

Las pipetas automáticas empleadas pertenecen a la casa comercial Thermo Scientific mod. Finnpipette, de volúmenes de 0,5-10 µL, 2-20 µL, 10-100 µL, 100-1000 µL y 1000- 5000 µL. Se han utilizado puntas sin filtro para los ensayos químicos y manejo de microorganismos de la marca Daslab®; y, para las técnicas moleculares y proteómica puntas con filtro Fisherbrand® Sure One de Fisher Scientific.

Para la conservación y manipulación de las muestras, en todos los pasos seguidos, se usaron microtubos de tapa con cierre seguro de distintos volúmenes (0,2, 0,5, 1,5 y 2 mL) de las marcas Daslab®, Axygen Scientific Y Deltalab.

La incubación de las bacterias a 37 °C se hizo en una estufa mod. 207 de P-Selecta.

La homogeneización de los microtubos tipo Eppendorf se realizó empleando un agitatubos Heidolph mod. Reax Top.

Las muestras de los microorganismos destinadas a su análisis mediante determinaciones moleculares fueron conservadas a una temperatura de -80 °C en un congelador Ult Freezer DW-86L628 de Haier Biomedical (-82 °C), hasta su utilización en los protocolos correspondientes. Los reactivos empleados en las técnicas laboratoriales que así lo requerían fueron almacenados a -20 °C en congeladores No Frost de Svan mod. SCV1863FFDX.

En los procedimientos de centrifugación se utilizó una centrífuga refrigerada mod. Centrifuge 5430R de Eppendorf, una centrífuga ST16R Sorvall de Thermo Scientific, así como una centrífuga mod. 5810 de Eppendorf.

En el protocolo de extracción de ARN, la incubación de las muestras a 65 °C se llevó a cabo en un termobloque mod. Dry Bath FB15101 de Fisher Scientific. Durante el proceso de extracción de ARN, así como en las técnicas de biología molecular y proteómica para mantener las condiciones óptimas de las muestras y reactivos químicos se utilizó hielo obtenido de una máquina de hielo triturado mod. IMS-85 Automatic Flake Ice Maker de Labreez.

La cantidad y calidad del ARN y de las proteínas extraídos fueron determinadas mediante un espectrofotómetro Thermo Scientific Nanodrop mod. 2000C de Thermo Fisher Scientific, conectado a un ordenador portátil Dell mod. Latittude D505.

Las mezclas de reacción de qPCR se realizaron en una campana Telstar mod. Mini-V/PCR. La retrotranscripción se llevó a cabo en un termocilador mod. Mastercycler® EP Gradient de Eppendorf.

Las reacciones de qPCR se realizaron con el sistema de qPCR Viia™ 7 de Applied Biosystems, conectado a un ordenador Dell mod. Optiplex XE. Además, se utilizaron placas de reacción de 96 pocillos (0,1 mL) MicroAmp™ Fast Optical con películas adhesivas MicroAmp™ de Applied Biosystems.

Para concentrar las muestras para los análisis químicos, se utilizó el Speed Vac Savant SPD131DDA de Thermo Scientific. Para obtener los lisados celulares para el análisis proteómico se utilizó una Prensa de French (Thermo Spectronic) con una presión aproximada de 68 atm (mini-cell de Thermo Spectronic)

Para el análisis del proteoma bacteriano se utilizó un espectrómetro de masas híbrido cuadrupolo Orbitrap™ Q-Exactive Plus (Thermo Scientific, Alemania) acoplado a un sistema de nanocromatografía de líquidos Ultimate 3000 RSLCnano (Thermo Scientific)

Los análisis de citometría de flujo se hicieron utilizando un citómetro a Cytoflex® (Beckman Coulter, EE. UU), equipado con láser violeta, azul y rojo.

Para la cuantificación de alisina, las muestras se llevaron a un HPLC Shimazu Prominence, equipado con un sistema de administración de solvente cuaternario (LC-20AD), un desgasificador en línea DGU-20AS, un muestreador automático SIL-20A, un detector de fluorescencia RF-10A XL y un controlador de sistema CBM-20A.

3.1.5. Software informático.

Los programas informáticos utilizados en esta Tesis Doctoral se presentan en la tabla 3.1.

Tabla 3.1: Programas informáticos utilizados en el desarrollo de esta Tesis Doctoral.

Programa	**Casa comercial**	**Finalidad**
Primer3 web v. 4.1.0	Elixir	Diseño de cebadores y sondas
ViiA™ 7 v. 1.2.4	Thermo Fisher Scientific	Manejo, desarrollo y obtención de los resultados obtenidos a partir de las reacciones de qPCR.
NanoDrop™ 2000/2000C v. 1.4.2	Thermo Fisher Scientific	Medida de la cantidad y calidad de los ácidos nucleicos
MaxQuant (v. 1.6.15.0)	Max Planck Institute of biochemistry	Para organizar los datos y realizar análisis estadísticos
ClueGO (v. 2.5.6) (Bindea et al., 2009).	Cytoscape	Para el análisis de enriquecimiento de las proteínas
Microsoft Excel 2016	Microsoft	Análisis de datos, elaboración de tablas y obtención de gráficos
Microsoft Power Point 2016	Microsoft	Elaboración y adaptación de figuras
SPSS Statistic v. 22	IBM	Tratamiento estadístico de los resultados

3.2. Métodos

3.2.1. Diseño experimental

Para la consecución de los objetivos de la Tesis Doctoral se siguió el diseño experimental indicado en la Figura 3.1.

Lactobacillus reuteri

Incubación del microorganismo, a 37 °C, en microaerofilia, con compuestos derivados de la oxidación de lípidos (MDA) y proteínas (AAA). Cuatro grupos de ensayo en cada caso:

- Malondhiadehído (MDA): Control, 5 µM, 25 µM y 100 µM
- Ácido aminoadípico (AAA): Control, 1 mM, 5 mM, 10 mM

Evaluación de la respuesta del microorganismo mediante la determinación de:

- Expresión génica de *uspA* y *dhaT*, genes relacionados con la respuesta a estrés.
- Análisis de la oxidación de proteínas:
  - Citometría de flujo,
  - Evaluación de la oxidación de lípidos y proteínas

¿El MDA puede producir carbonilos?

Incubación de proteínas humanas con MDA (0,25 mM):

- Seroalbúmina humana (HSA)
- Hemoglobina (HEM)
- B-lactoglobulina (LAC)

Enterococcus faecium

Incubación del microorganismo, a 37 °C, en anaerobiosis, con un compuesto oxidante (H_2O_2) y un compuesto fenólico con características antioxidantes (ácido clorogénico), cuatro grupos de ensayo:

- Control
- Ácido clorogénico (500 µM);
- Peróxido de Hidrógeno (2,5 mM);
- Ácido clorogénico (500 µM) + Peróxido de Hidrógeno (2,5 mM

Evaluación de la respuesta del microorganismo mediante el estudio de:

- Proteoma bacteriano
- Análisis de la oxidación de proteínas:
  - Citometría de flujo,
  - Evaluación de la oxidación de lípidos y proteínas

Figura 3.1. Diseño experimental de la presente Tesis Doctoral.

3.2.2. Condiciones de incubación del material biológico

En la evaluación de las respuestas de *Lactobacillus reuteri* frente a compuestos oxidantes, el microorganismo se incubó con distintas concentraciones de AAA y de MDA. En cada caso se hicieron cuatro grupos experimentales: con AAA: control (*L. reutri*), 1 mM (*L. reuteri* + 1 mM AAA), 5 mM (*L. reuteri* + 5 mM AAA), 10 mM (*L. reuteri* + 10 mM AAA). En el ensayo con MDA los grupos fueron: Control *(L. reuteri*), 5 µM (*L. reuteri* + 5 µM), 25 µM (*L. reuteri* + 25 µM) y 100 µM (*L. reuteri* + 100 µM).

Ambos ensayos se hicieron por triplicado para cada tratamiento. Los tubos, con un volumen final de 5 mL, con las diluciones de cada uno de los compuestos fueron inoculados con 100 µL de cultivo overnight de *L. reuteri* en caldo MRS y se cultivaron a 37 °C durante máximo 24 h. Las muestras fueron recogidas en cuatro tiempos: a las 12, 16, 20 y 24 h de incubación desde la inoculación.

Para análisis posteriores de determinación de proteínas las muestras se lavaron con tampón fosfato salino (PBS, pH 7,4) dos veces. Para el recuento de células viables se inocularon 100 µL de las muestras recogidas a cada tiempo y condiciones de los experimentos.

Por otro lado, para evaluar la capacidad de detoxificar productos de oxidación de *Enterococcus faecium* se incubó 1 mL de cultivo overnight en 240 mL de caldo MRS en cuatro grupos experimentales: control, ácido clorogénico (AC) (500 µM), peróxido de hidrógeno (H_2O_2; 2.5 mM) y con una mezcla de los dos compuestos. Todos los tratamientos se incubaron durante 48 h a 37 °C. Desde la inoculación, se tomaron muestras por triplicado a las 0, 12, 24 y 48 h para realizar ensayos de determinación de daño oxidativo. A las 48 h se tomaron las muestras para el análisis proteómico. El ácido clorogénico se diluyó en Dimetil Sulfóxido (DMSO), de manera que este compuesto se incluyó en todos los grupos de estudio.

Para evaluar como afecta cada tratamiento en el crecimiento de las bacterias ácido-lácticas se han realizado recuentos microbianos en placa mediante siembra en extensión en superficie y el conteo de las colonias viables. Se han utilizado placas de MRS agar previamente preparadas, donde se deposita 0,1 mL de muestra, previamente diluida en agua de peptona estéril, que se extiende con un asa de Digralsky estéril. Las placas fueron incubadas durante 24 h en condiciones de microaerofilia.

3.2.3. Estudio de la expresión génica relativa de genes de estrés oxidativo de BAL como respuesta a condiciones oxidantes.

3.2.3.1. Diseño de cebadores

Para llevar a cabo el estudio de las respuestas moleculares de *L. reuteri* frente a agentes oxidantes mediante qPCR con SYBR Green I, se utilizaron 2 parejas de cebadores para genes de estrés oxidativo.

Por un lado, el gen *uspA*, para el que se han diseñado cebadores siguiendo los requisitos para el diseño de cebadores de qPCR descritos por Rodríguez et al. (2015) y, por otro lado, el gen *dhaT* con los cebadores diseñados por Schaefer et al. (2010). Además, control endógeno se ha utilizado la amplificación de la subunidad 16S del ARNr de *L. reuteri*.

Los cebadores (Tabla 3.2) fueron diseñados utilizando el software Primer3Web (Untergasser et al., 2012) a partir de secuencias conocidas de los genes extraídas de la base de datos GenBank. Finalmente, para comprobar la especificidad de los cebadores diseñados, sus secuencias se compararon *in silico* con las secuencias de nucleótidos de la base de datos GenBank.

Tabla 3.2: Cebadores empleados en el estudio de la expresión génica

Gen	Cebadores	Secuencias de nucleótidos (5´- 3´)	Tamaño fragmento (pb)	Referencias
UspA	uspALr-F1	CTTGGGTAGCGTTCACCATT	81	Arcanjo et al., 2019
	uspALr-R1	TGAAAAAGCGGTTGACACTG		
dhaT	LS67	TGACTGGATCCTAATTTGGTCCTGGTGTTATTGC	81	Schaefer et al., 2010
	LS68	TGACTGAATTCTTCCGGATCTTAGGGTTAGG		
16S	Lr16S_F	CCGCTTAAACTCTGTTGTTG	79	Arcanjo et al., 2019
	Lr16S_R	CGTGACTTTCTGGTTGGATA		

3.2.3.2. Optimización de las condiciones de reacción.

Para llevar a cabo la optimización de las condiciones de reacción, en primer lugar, se realizó la extracción de ADN de *L. reuteri*, siguiendo las instrucciones del fabricante del kit comercial Master Pure Complete DNA and RNA Purification Kit, para lo cual se tomó 1 mL de cultivo puro de la bacteria (10^8 ufc/mL). El ADN extraído fue resuspendido en 35 µL de tampón TE y mantenido a -20 °C hasta su uso.

La concentración y pureza del ADN se determinaron por espectrofotometría. La relación de absorbancia A_{260}/A_{280}, determina la calidad del ADN, de esta manera, se considera que un ADN de buena calidad tiene un valor de entre 1,8 - 2,0 (Sambrook et al., 1989).

En la optimización de las condiciones de reacción se utilizó el ADN de *L. reuteri,* por triplicado, en un volumen final de 12,5 µL y en placas MicroAmp de 96 pocillos cubiertas por lámina óptica adhesiva. En todas las reacciones se incluyó un triplicado control con agua destilada estéril en lugar de ADN. Se utilizaron distintas concentraciones de cebadores, en un rango de 50 a 300 nM y unas condiciones de temperatura de hibridación y tiempos comprendidos entre los 55 y 60 °C; y desde 30 s a 1 min, respectivamente. Las condiciones finales de reacción se presentan en la Tabla 3.3.

Tabla 3.3: Condiciones de las reacciones de qPCR

Gen	**Condiciones**
uspA	1 ciclo 95 °C, 1º min 40 ciclos: 95 °C, 15 s y 60 °C, 1 min
dhaT	
16S	1 ciclo 95 °C, 1º min 40 ciclos: 95 °C, 15 s y 55 °C, 1 min

Al finalizar los ciclos de amplificación, se llevó a cabo el análisis de la curva de disociación del producto de PCR mediante temperaturas comprendidas entre los 60 y 99 °C, donde se obtuvieron medidas de fluorescencia de manera continua, calculándose de forma automática la temperatura de fusión o Tm. Posteriormente, se comparó el valor de Tm obtenido con el valor de Tm esperado para el producto de PCR amplificado y así determinar la especificidad de los cebadores diseñados.

Además, para evaluar la sensibilidad y eficiencia de las reacciones de qPCR optimizadas, se elaboraron curvas estándar con ADN de *L. reuteri*. Para ello se utilizaron cinco diluciones seriadas de ADN, con concentraciones de los 80 a los 0,0008 ng/ µL, aplicando las condiciones de reacción. Una vez obtenidos los valores de Ct, se relacionaron con las distintas concentraciones de ADN utilizadas, construyéndose así las curvas estándar correspondientes (Rodríguez y col., 2015). En este ensayo se utilizaron triplicados de cada dilución de ADN. Los criterios considerados para determinar la eficiencia de la reacción fueron el coeficiente de correlación (R^2) de las curvas estándar y la eficiencia de la reacción, calculada mediante la fórmula $E= 10^{-1/S}-1$, siendo S la pendiente de la curva estándar (Rodríguez et al., 2015).

3.2.3.3. Extracción de ARN

Para la extracción del ARN de *L. reuteri*, después de cada tiempo de incubación señalado en el diseño experimental, se tomó 1 mL de muestra de cada uno de los triplicados de cada tratamiento, que fue conservado a -80 °C hasta el momento de su utilización.

La extracción del ARN se hizo utilizando el kit comercial MasterPure™ RNA purification kit, siguiendo las instrucciones del fabricante, que incluye tratamiento con DNasa para eliminar contaminación con ADN genómico de la muestra. El ARN obtenido fue eluido en 35 µL de tampón TE, suministrado en el kit, y mantenido a -80 °C hasta su utilización.

La calidad y cantidad de ARN se midieron espectrofotométricamente mediante Nanodrop 2000 (Thermo Scientific), obteniendo las medidas de concentración (ng/µL) y pureza (ratio $A_{260/280}$). La relación de absorbancia $A_{260/280}$ determina que un ARN es de buena calidad cuando su valor se encuentra en torno a 2 (Sambrook et al., 1989).

3.2.3.4. Síntesis de ADNc

Antes de la síntesis de ADNc, se normalizó la concentración de ARN de todas las muestras a 100 ng/µL.

La síntesis de ADNc se hizo siguiendo las instrucciones del kit PrimeScript™ RT Reagent kit, partiendo de unos 500 ng de ARN y en un volumen final de 10 µL. Las condiciones de síntesis consistieron en un ciclo de 15 min a 37 °C, para el funcionamiento de la enzima de transcripción inversa y un ciclo de 5 s a 85 °C en el que se produce la inactivación de esta. El ADNc fue conservado a -20 °C hasta su uso. Previamente a su utilización en las reacciones de qPCR el ADNc fue diluido 1:10.

3.2.3.5. Análisis de la expresión génica

Para llevar a cabo el análisis de la expresión génica relativa de los genes de respuesta a estrés, *uspA* y *dhaT*, se utilizó la metodología SYBR Green™, relacionando la expresión de las muestras con una muestra control o calibrador y con el gen 16S del ARNr como control endógeno, ya que mantiene la expresión constante y se utiliza para normalizar las diferencias de concentración que pudieran existir.

Las reacciones de amplificación se hicieron por triplicado en un volumen final de 12,5 µL, con la composición indicada en la Tabla 3.4. Se incluyeron tres muestras control en las que había agua en lugar de ADNc.

Tabla 3.4: Composición de la mezcla de reacción

Gen	Cebador F (nM)	Cebador R (nM)	Sybr Green Mix (µL)	ROX (µL)	ADNc (µL)	Agua (µL)
uspA	300	300	6,25	0,625	2,5	2,375
dhaT	300	300	6,25	0,625	2,5	2,375
16S	300	300	6,25	0,625	2,5	2,375

Cebador F: Cebador Forward; Cebador R: Cebador Reverse

El análisis de los resultados obtenidos por qPCR se llevó a cabo por el método definido por Livak y Schmittgen (2001) del $2^{-\Delta\Delta Ct}$ o método de cuantificación relativa de los niveles de expresión de los genes diana respecto al gen constitutivo o control endógeno 16S. Se evaluó la eficiencia de las curvas estándar de los genes diana y del control endógeno se calculan y optimizan según se describe en el apartado 3.2.3.2.

3.2.4. Evaluación del proteoma de *E. faecium* frente a condiciones de estrés oxidativo y antioxidantes

Para determinar el mecanismo de acción de *E. faecium* para detoxificar agentes oxidantes se realizó un análisis de su proteoma. Para ello se partió de 200 mL de cultivo de los grupos experimentales descritos en el apartado 3.2.1, por triplicado, las células se recogieron por centrifugación a 6.000 rpm durante 10 min a 4°C en una centrífuga Avanti TM J-25 Beckman Coulter con un rotor JA-14. El sobrenadante se desechó y el precipitado se disgregó en 4 mL de tampón de lisis (Tris-HCl 100 mM, NaCl 50 mM, EDTA 20 mM, 10 % glicerol, PMSF 1 mM y pepstatina A 1 µg/µL; pH 7,5). Las células recogidas se sometieron a rotura por diferencia de presión mediante la utilización de la Prensa de French con una presión aproximada de 68 atm (mini-cell). El resultado se centrifugó durante 15 minutos, 12.000 rpm, 4°C, para eliminar restos celulares y células sin romper.

Después de la lisis celular, la extracción de proteínas en los sobrenadantes se llevó a cabo mediante el protocolo descrito por Delgado et al. (2015), que consiste en precipitar las proteínas con ácido tricloroacético (TCA) y lavados con acetona (Carpentier et al., 2015).

Los lisados precipitados fueron resuspendidos en tampón de urea 8 M y se midió su concentración con Coomasie Protein Assay Reagent Ready to Use en el espectrofotómetro Nanodrop 2000c para garantizar cantidades de proteínas homogéneas en las diferentes muestras.

Cinco alícuotas por tratamiento (de las que tres eran replicados biológicos y dos técnicos) con 50 µg fueron tratados según el protocolo descrito por Delgado et al., (2015) (a y b) y Owens et al., (2015). Que consiste en que las muestras fueron incubadas con 1,4- ditiotreitol (DTT) 0,5 M en bicarbonato de amonio 50 mM durante 20 min a 56 °C, para la reducción proteica. Los grupos tiol (-SH) resultantes se sometieron a alquilación mediada por iodoacetamida 0,55 M en bicarbonato de amonio 50 mM durante 15 min en oscuridad a temperatura ambiente. Para la obtención de péptidos se añadieron tripsina de grado de secuenciación (Promega, España) y el surfactante ProteaseMAX (Promega, España), siguiendo las instrucciones del fabricante y, finalmente se añadió 1 µL de ácido fórmico 100% para parar la reacción de proteólisis. El sobrenadante se secó a vacío y las muestras digeridas se sometieron a un protocolo para eliminar las sales presentes utilizando las puntas Pierce™ C18 (Thermo Scientific).

Antes de analizar las muestras en el Orbitrap LC-MS/MS, los péptidos resultantes de la digestión tríptica se resuspendieron en tampón de carga (98% de agua milli-Q, 2% de acetonitrilo, 0,05% de ácido trifluoroacético), se sonicaron en baño de agua durante 5 min y se centrifugaron a 14.452 g durante 15 min a temperatura ambiente y se transfirieron a viales para el Oribrtrap LC-MS/MS.

Se analizaron 2 µg de cada digestión en un espectrómetro de masas Q-Exactive Plus acoplado a un Dionex Ultimate 3000 RSLCNano (Thermo Scientific). Los datos se recogieron utilizando un método dependiente de datos Top15 para escaneos MS/MS (Delgado et al., 2019).

La comparación entre proteomas y el análisis de datos se realizaron utilizando el software MaxQuant (v. 1.6.0.15.0; test https://www.maxquant.org/download_asset/maxquant/la) y Perseus (v 1.6.14.0) para organizar los datos y realizar análisis estadístico.

La carbamidometilación de cisteínas se estableció como una modificación fija, mientras que la oxidación de metioninas y la acetilación de N-terminales se establecieron como modificaciones variables. La búsqueda en la base de datos se realizó en una base de datos de proteínas de *E. faecium* descargada de Uniprot (https://www.uniprot.org/). Las tasas máximas de falso descubrimiento de péptidos/proteínas (FDR) se establecieron en 1% en base a la comparación con una base de datos inversa. El algoritmo cuantitativo sin marcaje (LFQ) se utilizó para generar intensidades espectrales normalizadas e inferir la abundancia relativa de proteínas (Luber et al., 2010). Las proteínas fueron

identificadas con al menos dos péptidos y aquellas que coincidían con una base de datos de contaminantes o la base de datos inversa, se eliminaron, y solo se conservaron en el análisis final si se detectaban en al menos dos réplicas de al menos un tratamiento. El análisis cuantitativo se realizó usando una prueba t para comparar los tratamientos con el control (p <0.05). También se realizó un análisis cualitativo para detectar proteínas encontradas en al menos tres réplicas de un tratamiento en particular, pero indetectables en el tratamiento comparado. Todas las proteínas que cumplían con uno de estos dos criterios fueron identificadas como "proteínas discriminantes".

3.2.5. Análisis de la oxidación de proteínas

3.2.5.1. Citometría de flujo

El análisis mediante citometría de flujo para analizar las células viables y la generación de ROS por parte de las bacterias se llevó a cabo siguiendo el protocolo previamente descrito por Ortega-Ferrusola et al., (2017) y Peña et al., (2018).

Para llevar a cabo el análisis de citometría de flujo se utilizan sondas de tinción que se unen a elementos específicos de la célula, según lo que se quiera conocer. De esta manera, el equipo diferencia las células en subpoblaciones en función de la cantidad detectada de cada componente.

Los cultivos bacterianos en una concentración de $1x10^6$ (ufc/mL), fueron resuspendido en 1 mL de PBS y teñidos con dos sondas de tinción:

- CellRox Deep Red (5 µM), detecta la cantidad de bacterias productoras de ROS como consecuencia del estrés oxidativo. Las longitudes de onda de excitación y emisión son de 644 nm y 665 nm, respectivamente.
- Hoechst 33342 (0,5 µM), identifica la cantidad de ADN que hay en la muestra, es decir, identifica las bacterias viables. Longitudes de onda de excitación y emisión de 345 nm y 488 nm, respectivamente.

Tras añadir las sondas, se mezcló suavemente y la suspensión de células fue incubada a temperatura ambiente en oscuridad durante 25 min, tras lo que se lavó con PBS y se introdujeron inmediatamente en el citómetro de flujo. Se utilizaron controles sin tinción, tinción simple y de fluorescencia menos uno (FMO) para determinar compensaciones y eventos positivos y negativos, así como para establecer regiones de interés.

El instrumento fue calibrado diariamente utilizando soluciones de calibración específicas proporcionadas por el fabricante. Antes de cada experimento, se realizó una superposición de emisión

y excitación, sin embargo, debido a las características de emisión y excitación de las sondas utilizadas, la superposición espectral fue insignificante. Los archivos se exportaron como FCS y se analizaron con el software Flowjo v. 10.5.3 para Mac OS (Ashland).

3.2.5.2. Cuantificación de alisina

Para la cuantificación de alisina se realizó siguiendo el método descrito por Villaverde & Estévez, (2013), en el que se partió de 500 µL de cultivo, que fueron tratados con una solución de TCA frío al 10%. Cada muestra fue centrifugada a 600 g durante 5 min a 4 °C. Los sobrenadantes se eliminaron y los pellets se incubaron con soluciones recién preparadas de: 0, 5 mL de tampón ácido 2- (N-morfolino) etanosulfónico (MES) 250 mM, pH 6.0, que contenía 1 mM de ácido dietilentriaminopentaacético (DTPA); 0,5 mL de ABA 50 mM en tampón MES, pH 6.0; y 0.25 mL de $NaBH_3CN$ 100 mM en 250 mM de tampón MES, pH 6.0.

Los microtubos se agitaron y se incubaron en un baño de agua a 37 °C durante 90 min, las muestras fueron agitadas cada 15 min. Después, las muestras se trataron con una solución de TCA 50% a 4 °C y centrifugadas a 1200 g 10 min. Los pellets se lavaron dos veces con TCA 10% y dietiléter- etanol (1:1, v/v). Finalmente, el sedimento fue tratado con 6N de HCl e incubado a 110 °C, durante 18 h hasta completar la hidrólisis. Los hidrolizados fueron secados a vacío en un speed-vac. El residuo generado en el microtubo fue reconstituido con 200 µL de agua milliQ y filtrado a través de filtros de jeringa hidrofílicos de polipropileno GH Polypro (GHP) de 0.45 µM de tamaño de poro (Pall Corporation, New York, NY, EE. UU.) para el análisis por HPLC.

A continuación, 1 µL de los hidrolizados proteicos de las muestras se inyectó en el HPLC Shimazu Prominence, equipado con un sistema de administración de solvente cuaternario (LC-20AD), un desgasificador en línea DGU-20AS, un muestreador automático SIL-20A, un detector de fluorescencia RF-10A XL y un controlador de sistema CBM-20A, para su análisis. AAS- ABA fueron eluidos en una columna Cosmosil 5C18-AR-II RP-HPLC (5 µm, 150 x 4,6 mm) equipada con una precolumna (10 x 4,6 mm) empaquetada en el mismo material. El flujo y la temperatura de la columna se mantuvieron constantes a 1 mL/min y a 30 °C, respectivamente. Los compuestos eluidos se monitorizaron con longitudes de onda de excitación y emisión fijadas en 283 y 350 nm, respectivamente. Se utilizaron compuestos estándar (0,1 µL) que fueron analizados en las mismas condiciones. La identificación de los semialdehídos derivatizados en los cromatogramas se llevó a cabo comparando los tiempos de retención con los de los compuestos estándar. El pico correspondiente a la alisina-ABA fue integrado manualmente a partir de los cromatogramas y las áreas resultantes se compararon con una curva

estándar de ABA con concentraciones conocidas que oscilaron entre 0,1 y 0,5 mM. Los resultados se expresaron como nmol de alisina por mg de proteína.

3.2.5.3. Estudio in vitro *de la reactividad de MDA con proteínas*

Para evaluar la capacidad del MDA de inducir la carbonilación de proteínas se han utilizado 3 proteínas: albúmina de suero humano (HSA), hemoglobina humana (HH) y β-lactoglobulina (LAC) en concentración final de 5 mg/mL, que fueron disueltas en 100 mM de tampón fosfato pH 6,5 e incubadas por separado con 0,25 µM de MDA a 37 °C (HSA, HH) y a 80 °C (LAC) en un horno con agitación constante durante 24 h. Las proteínas fueron seleccionadas según su susceptibilidad al daño oxidativo (Luna y Estévez, 2018; Arcanjo y col., 2018).

Las muestras se tomaron a las 24 h para la cuantificación de alisina y de Bases de Schiff. Se prepararon seis grupos experimentales que corresponden a las parejas experimentales: HSA-MDA, HH-MDA y LAC-MDA y sus correspondientes controles (proteínas sin MDA), se hicieron tres replicados en ensayo independientes y todos los análisis se repitieron tres veces en cada pareja experimental (9 mediciones por análisis y por tratamiento para calcular medias y desviaciones estándar).

3.2.5.4. Análisis de las Bases de Schiff

La formación de bases de Schiff se determinó utilizando el espectrómetro de fluorescencia LS-55 Perkin-Elmer (PerkinElmer, UK). Antes del análisis, las muestras se diluyeron 1:20 con tampón de urea 8 M en 100 mM de tampón fosfato de sodio pH 7. Las bases de Schiff fueron excitadas a 350 nm y la fluorescencia emitida fue recogida a 450 nm. La variación de excitación y emisión se fijó en 10 nm y la velocidad de la recogida de datos mientras se hacía el análisis fue de 500 nm por minuto. Se recogió la altura de los picos correspondientes con las bases de Schiff. Tras tener en cuenta las diluciones aplicadas, los resultados se expresaron como unidades de fluorescencia.

3.2.5.5. Análisis de tioles

Para evitar la posible contaminación con tioles contenidos en el medio de cultivo, se tomaron 250 µL de cada muestra que fueron lavados dos veces con PBS y con etanol: acetato de etilo (1:1). El pellet fue resuspendido en 250 µL de clorhidrato de guanidina y añadido a la cubeta en un volumen final de 1250 µL de clorhidrato de guanidina. La absorbancia fue medida a 324 nm antes y después de añadir 250 µL de disulfuro de 4,4'dipiridilo (4-DPS) en 12 mM de HCl. Los resultados obtenidos fueron expresados como µmol de grupos tioles libres por mg de proteína.

3.2.5.6. Análisis de sustancias reactivas al ácido tiobarbitúrico

El MDA y otras sustancias reactivas al ácido tiobarbitúrico (TBARS) se midieron a partir de 200 µL de cada muestra, añadiendo 500 µL de ácido tiobarbitúrico (TBA) (0.02 M) y 500 µL de TCA (10%), que fue incubado durante 20 min a 90 °C. Tras dejarlo enfriar, se centrifugó 5 min a 600 g y el sobrenadante se midió a 532 nm. Los resultados se expresan como mg de TBARS por L de muestra.

3.2.5.7. Cuantificación de peróxido de hidrógeno.

El peróxido de hidrógeno (H_2O_2) fue cuantificado en *E. faecium* utilizando el método descrito por Jiang et al (1990) con algunas modificaciones. Las muestras de *E. faecium* ($1x10^6$ ufc/mL) tomadas a las 12, 24 y 48 h fueron diluidas en una mezcla de hexano e isopropanol (3:1, v/v). Se mezcló y dispensó en cubetas de cuarzo para medir la absorbancia a 240 nm en un espectrofotómetro Shimadzu 1800. Se prepararon curvas estándar con H_2O_2 para cuantificar utilizando el coeficiente de extinción 43.6 M^{-1} * cm^{-1} a 240 nm. Los datos se expresan como pM de H_2O_2.

3.2.5.8. Análisis de la actividad tipo catalasa

La capacidad de *E. faecium* de decomponer H_2O_2 se llevó a cabo mediante el procedimiento publicado por Li & Schellhorn (2007) con modificaciones. En resumen, se tomaron muestras de *E. faecium* ($1x10^6$ ufc/mL)) a las 48h, que se expusieron a una solución de H_2O_2 y se dejaron a temperatura ambiente (22°C) durante 180 s. Cada 30 s se evaluó el agotamiento de H_2O_2, midiendo la absorbancia a 240 nm en un espectrofotómetro Shimadzu 1800. Se prepararon curvas estándar con H_2O_2 para cuantificar utilizando el coeficiente de extinción 43.6 M^{-1} * cm^{-1} a 240 nm. Los datos se expresan como pmol agotados de H_2O_2/min*mL.

3.2.5.9. Análisis de carbonilos por el método DNPH

La cantidad de carbonilos totales se determinó en muestras de *E. faecium* tomadas a las 48 h mediante el método de dinitrofenilhidrazina (DNPH) como describen Estévez et al., (2008) con modificaciones. En resumen, se precipitaron proteínas de *E. faecium* ($1x10^6$ ufc/mL) mediante la adición de 1 mL de ácido tricloroacético (TCA) al 10% frío, seguido de centrifugación a 4°C a 600 g durante 5 min. Los sedimentos de proteínas se trataron con 1 mL de una solución de HCl 2 M con DNPH al 0,2% y se incubaron a temperatura ambiente durante 1 h. Las proteínas se precipitaron posteriormente con 1 mL de TCA al 10% frío, seguido de centrifugación a 4°C, 1200 g durante 10 min y se lavaron dos veces con 1 mL de etanol: acetato de etilo (1: 1 v / v). Los sedimentos se disolvieron en 1.5 mL de tampón Na_3PO_4 20 mM pH 6.5 y se les añadió clorhidrato de guanidina hasta 6 M. La cantidad de carbonilos se

expresó en nmoles de hidrazonas proteicas por mg de proteína utilizando un coeficiente de extinción molar de hidrazonas de 21.0 nM^{-1} cm^{-1} a 370 nm.

3.2.5.10. Análisis de grupos amino libres.

Los grupos amino libres se cuantificaron como describen Weigele et al., (1972) y Strauss & Gibson (2004). Las suspensiones de proteínas (850 µl) se añadieron a 2 mL de tetraborato de sodio 0.05 M (pH 8,5) en una cubeta de espectrofluorómetro de cuarzo de 4 mL. Posteriormente, se dispensaron 150 µL de solución de fluorescamina 0,7 mM en acetona. La cubeta se invirtió cuatro veces y la fluorescencia resultante se midió usando 390/485 nm para excitación y emisión en un espectrómetro de fluorescencia Perkin Elmer LS45 (Llantrisant, Reino Unido). La concentración de grupos amino libres se calculó basándose en una curva estándar preparada a partir de lisina diluida en tampón de ácido málico (pH 5.8). La contribución del tampón de ácido málico (pH 5.8) se registró en las mismas condiciones y se restó de todas las muestras. La concentración se expresa en µmol de grupos amino/mg de proteína.

3.3. Tratamiento estadístico

En los ensayos con *L. reuteri* el análisis estadístico se realizó, tras la recogida de datos, en primer lugar, mediante la prueba de Saphiro-Wilk con la que se determinó si los datos obtenidos seguían una distribución normal. A los datos que seguían una distribución normal se les aplicó un análisis de la varianza (ANOVA) para determinar las diferencias significativas entre las medias. El efecto de MDA y AAA sobre la expresión génica (valores de ΔΔCT) se analizó mediante pruebas t de Student pareadas (SPSS v. 15.5). La significancia estadística se estableció en $p \leq 0,05$.

En el ensayo llevado a cabo con *E. faecium*, todos los experimentos se realizaron 5 veces (3 réplicas biológicas + 2 réplicas técnicas). Para la técnica de citometría de flujo cada muestra fue analizada dos veces. Se analizó la normalidad y homocedasticidad de los resultados. El efecto de la exposición a H_2O_2 y a AC se evaluó mediante el Análisis de Varianza (ANOVA). Las comparaciones múltiples de medias se realizaron mediante un test de Tukey, el efecto del tiempo de incubación sobre las mismas medias seevaluó mediante la prueba t de Student. El nivel de significación de fijó en $p<0,05$.

En ambos ensayos se utilizó SPSS (v.15.0) para el análisis estadístico de los datos.

4. RESULTADOS

4. RESULTADOS

4. I: Molecular mechanisms of the disturbance caused by malondialdehyde on probiotic *Lactobacillus reuteri* PL503.

4. II: Malondialdehyde Interferes with the Formation and Detection of Primary Carbonyls in Oxidized Proteins.

4. III: An in vitro assay of the effect of lysine oxidation end-product, α-aminoadipic acid, on the redox status and gene expression in probiotic *Lactobacillus reuteri* PL503.

4. IV: Chlorogenic acid modulates the antioxidant response of *Enterococcus faecium* to oxidative stress: A flow cytometry and proteomic study

4.I. Molecular mechanisms of the disturbance caused by malondialdehyde on probiotic *Lactobacillus reuteri* PL503.

Mecanismos moleulares de la alteración provocada por el malondialdehído en el probiótico *Lactobacillus reuteri* PL503.

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Molecular mechanisms of the disturbance caused by malondialdehyde on probiotic *Lactobacillus reuteri* PL503

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Summary

This study aimed to provide insight into the molecular and genetic mechanisms implicated in the responses of *Lactobacillus reuteri* against the oxidative stress induced by malondialdehyde (MDA) by analysing protein oxidation and assessing the *uspA* and the *dhaT* genes. Four experimental groups were evaluated depending on the concentration of MDA added in Man, Rogosa and Sharpe (MRS) broth: Control (*L. reuteri*), 5 μM (*L. reuteri* + 5 μM MDA), 25 μM (*L. reuteri* + 25 μM MDA) and 100 μM (*L. reuteri* + 100 μM MDA). Three replicates were incubated at 37 °C for 24 h in microaerophilic conditions and sampled at 12, 16, 20 and 24 h. The upregulation of the *uspA* gene by *L. reuteri* indicates the recognition of MDA as a potential DNA-damaging agent. The *dhaT* gene, encoding a NADH-dependent-oxidoreductase, was also upregulated at the highest MDA concentrations. This gene was proposed to play a role in the antioxidant response of *L. reuteri*. The incubation of *L. reuteri* with MDA increased the production of ROS and caused thiol depletion and protein carbonylation. *L. reuteri* is proposed to detoxify pro-oxidative species while the underlying mechanism requires further elucidation.

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Introduction

Oxidative stress is a redox deregulation typically caused by an imbalance between pro-oxidants and the antioxidant defences, which is manifested as damage to molecules of biological significance by radical and non-radical species (Finkel and Holbrook, 2000). The biological consequences of the oxidative damage to lipids, proteins and the DNA, involve impaired physiological processes and the onset of assorted health disorders. Profuse literature supports the implication of persistent oxidative stress on severe health disorders such as diabetes (Asmat *et al*., 2016), neurodegenerative and cardiovascular diseases (Lin and Beal, 2006), and several types of cancer (Valko *et al*, 2005; Reuter *et al*, 2010). The colon has been identified to be particularly sensitive to oxidative stress (Sanders *et al*., 2014) and such condition is involved in the onset of a number of pathological conditions at this location, including ulcerative colitis, Crohn's and inflammatory bowel diseases, and colorectal cancer (Gackowski *et al*., 2002; Zhu and Li, 2012). The intestinal mucosa is permanently exposed to the pro-oxidant action of dietary lipid and protein oxidation products (~ luminal oxidative stress) which may contribute to impair the redox status of the bowel (Estévez and Luna, 2017). Malondialdehyde (MDA), a remarkable lipid oxidation product in muscle foods and oxidized oils, is able to bind to DNA leading to the formation of etheno-modified DNA bases, with these adducts being found in organs with diseases related to enduring inflammatory conditions that may eventually cause malignancies (Bartsch and Nair, 2005). Owing to the pathological effects of luminal oxidative stress, dietary antioxidants have been proposed to inhibit and/or alleviate the symptoms of some of these health disorders (Spyropoulos *et al*., 2011). On this line, supplementation with probiotic bacteria has been proposed as a feasible strategy to counteract the oxidative stress in the gastrointestinal tract (GIT) in humans (Spyropoulos *et al*., 2011).

Probiotics are defined as 'live microbes which, when administered in adequate amounts, confer a health benefit to the host' (FAO, 2002). As a natural colonizer of the GIT tract, *Lactobacillus reuteri* has been widely used as a dietetic supplement to promote gut health in humans (Hjern *et al*., 2020; Wang *et al*., 2020). Oral

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administration of *L. reuteri* decreases the onset and severity of inflammatory and infectious disorders in the GIT and contributes to a balanced colonic microbiota (Wang *et al*., 2020). In relation to the underlying mechanisms of its probiotic effects, *L. reuteri* has been found to diminish colonic oxidative stress by reducing the formation and accumulation of oxidation products in the lumen (Amaretti *et al*, 2013). The positive effects of *L. reuteri* on the oxidative and health status of human colon are documented (Petrella, 2016) and include protection against a number of pathological conditions such as colorectal cancer (Bistas *et al*, 2020), inflammatory bowel diseases (Wang *et al*, 2020) and cardiovascular disorders (Kazemian *et al*, 2020). Yet, the precise biochemical and genetic responses of *L. reuteri* against prooxidative conditions caused by luminal oxidation products (such as MDA) are not fully understood.

Pathogenesis of MDA not only involves the formation of DNA adducts, this lipid peroxidation product is known to bind to proteins and induce a number of oxidative modifications (Esterbauer *et al*, 1993). From a medical standpoint, the accumulation of oxidized proteins is a pathological hallmark of ageing and chronic diseases (Estévez and Luna, 2017) and protein oxidation is known to play central role in the pathogenesis of gut disorders driven by oxidative stress (Gackowski *et al*, 2002; Zhu and Li, 2012). Though much less studied than in humans, the occurrence of protein oxidation in bacteria has also been linked to impaired growth and senescence (Ezraty *et al*., 2017). On this line, the modifications induced by MDA in bacterial proteins and their biological consequences, are unknown. Furthermore, the precise biological mechanisms activated by *L. reuteri* in response to an MDA-induced oxidative stress are poorly documented. In this regard, the study of the regulation of particular stress-related genes seems crucial to comprehend the influence of external sources of oxidative stress on particular metabolic pathways and biological functions. Previous reports have documented that particular genes such as *uspA* and the *dhaT* are activated in *L. reuteri* in response to an oxidative threat caused by reactive oxygen species (ROS; Arcanjo *et al*, 2019). The universal stress protein A (*uspA*) superfamily is an ancient and conserved group of proteins found in assorted microorganisms, insects and plants (Kvint *et al*., 2003). The precise roles of *Usp* proteins in biological systems remain unclear; yet, they seem to be involved in the defence against DNA-damaging agents (Kvint *et al*, 2003). The *dhaT* gene expression leads to the synthesis of a 1,3 propanediol oxidoreductase (1,3-PDO), which was proposed by Arcanjo *et al*. (2019) to be involved in the protection of *L. reuteri* against oxidative stress. Arcanjo *et al*. (2019) also reported that protein carbonylation could play a relevant role as indicator of oxidative damage in bacteria and as a potential signalling mechanism. Yet, the connection between oxidative stress, gene expression and protein oxidation in probiotic bacteria is poorly understood.

This study aimed to provide insight into the molecular and genetic mechanisms involved in the responses of *L. reuteri* against MDA-induced oxidative stress by analysing protein oxidation and assessing the *uspA* and the *dhaT* genes.

Results

Regulation of uspA *and* dhaT *genes by* L. reuteri *in response to MDA*

Fig. 1A shows the relative expression ($2^{-\Delta\Delta C_T}$) of the *L. reuteri* PL503 *uspA* gene during the incubation assay in the presence of different concentrations of MDA. The exposure to MDA caused an upregulation of the *uspA* gene, as shown in Fig. 1A. This event had a dose-effect (12 h) which affected not only the intensity of the gene overexpression but also the time-frame of the occurrence of such biological response. The lowest concentration (5 μM) apparently led to a fast and limited response already at 12 h. The relative expression of the *uspA* gene in *L. reuteri* PL503 incubated with 25 and 100 μM of MDA showed a similar response 4 h later, while such expression had a peak after 20 h of incubation being significantly more intense with 25 and 100 μM of MDA than in the absence or 5 μM of MDA.

Overall, no significant changes in the relative expression of the *dhaT* gene were observed during the first two samplings. Yet, a dose-dependent effect of the incubation with MDA on the *dhaT* gene expression was found at 20 h of incubation (Fig. 1B). In particular, the relative transcription of the *dhaT* gene significantly increased in the presence of 25 and 100 μM of MDA at 20 h. At the final sampling, the relative expression of the *dhaT* gene significantly increased in the bacterium challenged with the highest concentration of MDA.

Induction of oxidative stress in L. reuteri *by MDA*

To investigate the ability of MDA to induce oxidative stress in *L. reuteri* PL503, ROS generation was assessed in bacteria by flow cytometry. Figure 2 shows how MDA exposure increased the percentage of bacteria suffering from oxidative stress in a dose-dependent manner. To assess the oxidative damage caused by MDA incubation in *L. reuteri* PL503, both lipid and protein oxidation markers were quantified.

The initial concentration of TBARS found in the cultures accurately reflects the three levels of MDA used for challenging *L. reuteri* PL503 (Fig. 3A). The basal TBARS concentration in CONTROL cultures

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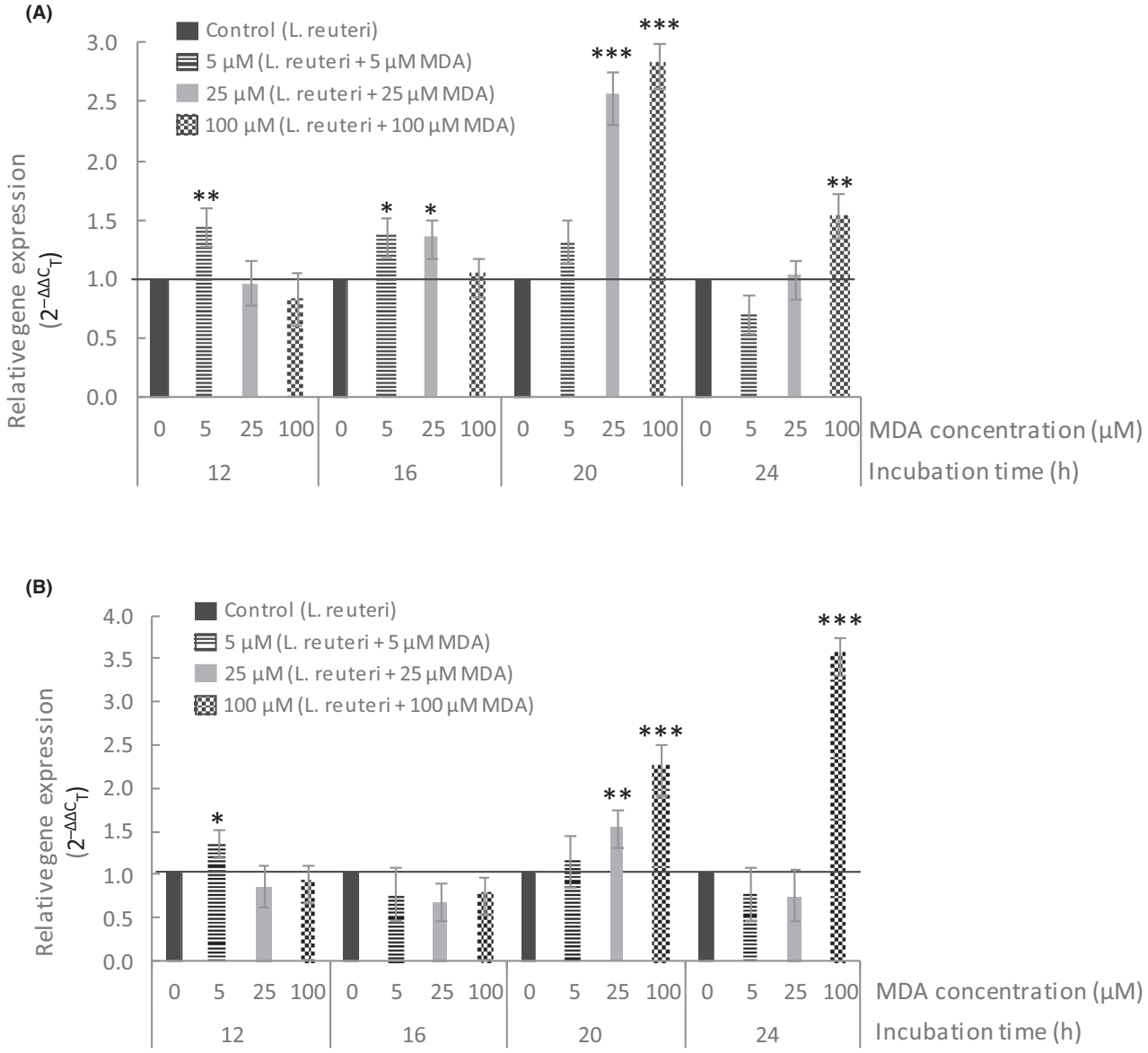

Fig. 1. Relative expression ($2^{-\Delta\Delta C_T}$) of the *uspA* (A) and *dhaT* (B) genes in *Lactobacillus reuteri* PL503 as affected by increasing concentrations of malondialdehyde (MDA) for up to 24 h. Means for each experimental group are calculated from analyses applied to three biological replicates and all analyses were technically duplicated. Black line at $2^{-\Delta\Delta C_T} = 1$ denotes standardized expression rate for CONTROL group at each sampling time (calibrator). $2^{-\Delta\Delta C_T} < 1$ denotes suppression of gene expression; $2^{-\Delta\Delta C_T} > 1$ denotes activation of gene expression. Asterisks on top of bars denote significant diferences in paired Student's *t*-tests performed to compare each MDA concentration with CONTROL [MDA = 0]: $*P \leq 0.05$; $**P \leq 0.01$; and $***P \leq 0.001$.

(~ 1 mg l^{-1}) could be the result of the occurrence of lipid peroxidation in the bacterium under physiological conditions and did not change significantly during the assay ($P > 0.05$). In the cultures challenged with MDA, the initial concentration significantly decreased ($P < 0.05$) in the range of 22–26% with this depletion being a likely reflection of the reaction of MDA with other biomolecules.

The evolution of the concentration of allysine, a recognized marker of protein oxidation, was assessed in *L. reuteri* PL503 during the incubation period (37 °C/24 h) and results are shown in Fig. 3B. The evolution of allysine in CONTROL group of *L. reuteri* PL503 shows a significant increase ($P < 0.05$) from 0.7 to 1.65 nmol allysine per mg protein. As compared to CONTROL, the exposure to MDA caused a significant increase ($P < 0.001$) in the concentration of allysine in proteins from *L. reuteri* PL503 and such increase followed a dose-dependent fashion for 16 h, suggesting a direct implication of MDA in the carbonylation of proteins from *L. reuteri* PL503. After that sampling, the behaviour varied between experimental groups. In *L. reuteri* PL503

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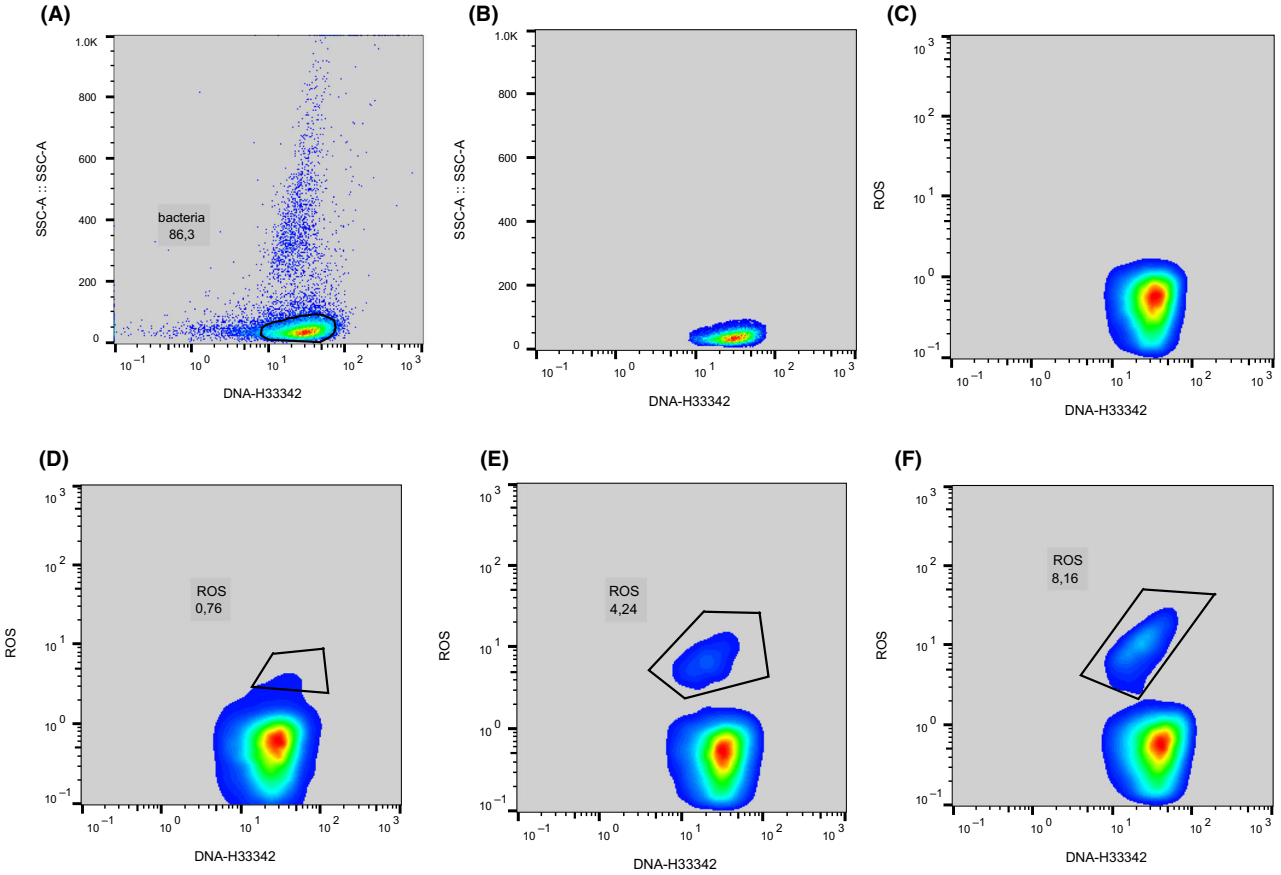

Fig. 2. Production of reactive oxygen species (ROS) in *Lactobacillus reuteri* PL503 grown in MRS broth with increasing concentrations of malondialdehyde (MDA) for 24 h. A: DNA containing bacterium (debris and nonbacterium events are removed); B: Gate for bacterium; C-F: X-axis bacterium (DNA +) Y-axis ROS; C: CONTROL; D: *L. reuteri* PL503 incubated with 5 μM MDA; E: *L. reuteri* PL503 incubated with 25 μM MDA; F: *L. reuteri* PL503 incubated with 100 μM MDA. Analyses were applied to three biological replicates and all analyses were technically duplicated.

grown in the presence of 5 and 25 μM of MDA, the increase of allysine was sustained during the complete assay reaching the highest concentration at 24 h (3.7 and 5.2 nmol allysine/mg protein respectively). When exposed to the highest MDA concentration (100 μM) the concentration of allysine peaked at 16 h after which a decrease was observed so that basal levels (< 1 nmol allysine mg^{-1} protein) were found at the end of the incubation period. In the present study, the formation of Schiff bases (Fig. 3C) was dependent on the presence of MDA. Yet, it is worth noting that no clear dose-dependent effect was observed and that the evolution of its concentration during the assay was erratic.

To verify whether MDA is able or not to induce allysine formation via an oxidative deamination mechanism similar to that exerted by other dicarbonyls (i.e. glyoxal), MDA (0.25 μM) was incubated with assorted proteins (5 mg ml^{-1}) at 37 °C (HSA, HH) and 80 °C (LAC) for 24 h. As compared to CONTROL groups (protein suspensions with no added MDA), protein suspensions incubated with MDA had significantly lower concentrations of allysine (Fig. 4A). Conversely, MDA caused a significant increase in the concentration of Schiff bases (Fig. 4B).

The concentration of free thiols in *L. reuteri* PL503 during the incubation assay (37 °C/24 h) is shown in Fig. 5. No significant changes in the thiol concentration were observed in CONTROL samples during the first 12 h. During the second half of the assay, a quantitatively small but significant increase of thiols was detected. The incubation of *L. reuteri* PL503 in the presence of MDA caused a significant dose-dependent increase of free thiols during the first 16 h of assay. In the following sampling points, the evolution of thiols remained stable in bacteria treated with 100 μM of MDA and significantly declined in bacteria exposed to 5 and 25 μM of MDA.

Discussion

Regulation of uspA *and* dhaT *genes by* L. reuteri *in response to MDA*

The increasing applied doses of MDA (5 μM, 25 μM, 100 μM) did not compromise the survival of *L. reuteri*, as

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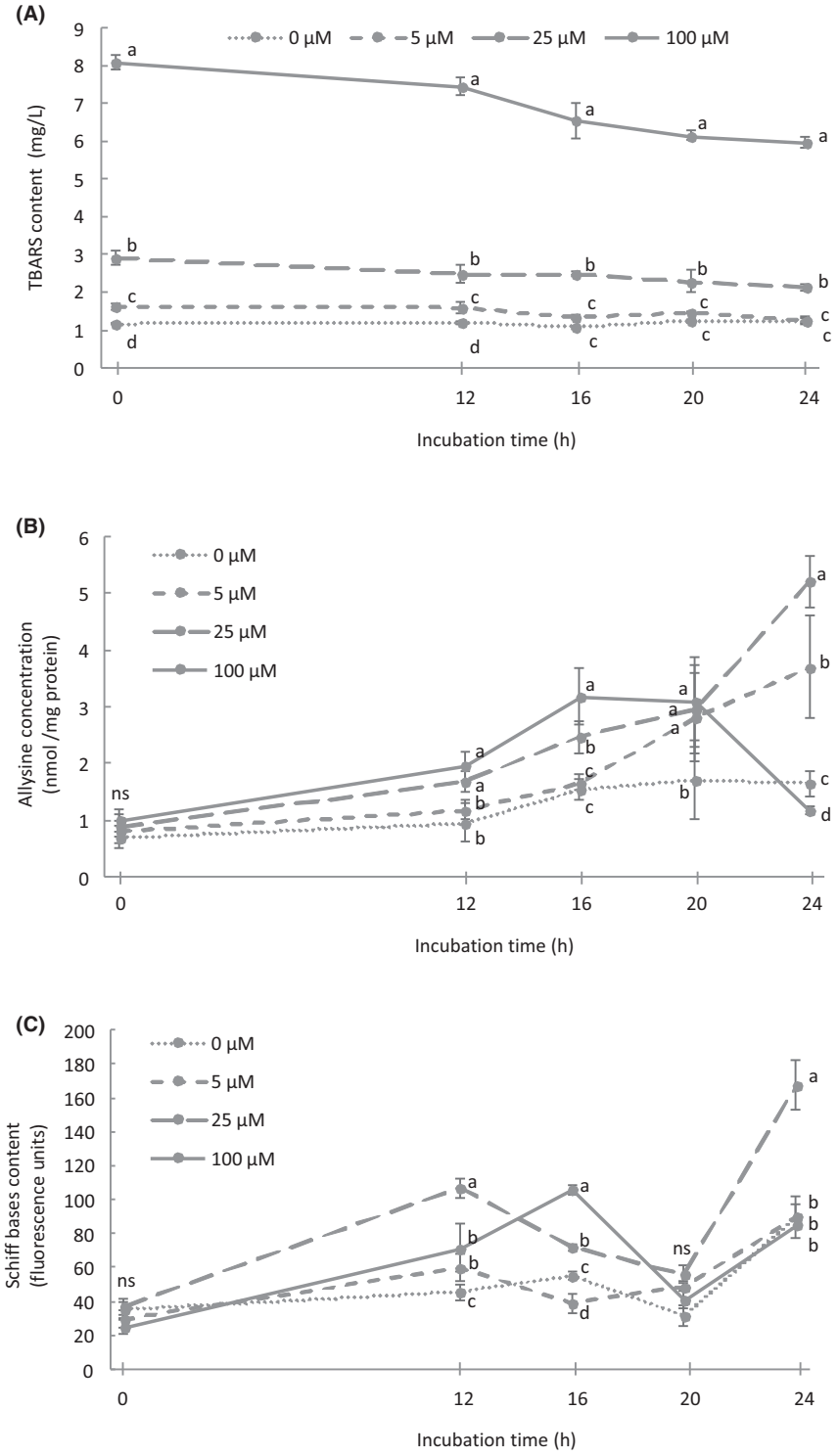

Fig. 3. Concentration of TBARS (A); allysine (B) and Schiff bases (C) (means ± standard deviation) in *Lactobacillus reuteri* PL503 grown in MRS broth with increasing concentrations of malondialdehyde (MDA) during an incubation period for up to 24 h. Means for each experimental group are calculated from analyses applied to three biological replicates and all analyses were technically duplicated. Different letters at the same sampling time denote significant differences between means within the same sampling point in ANOVA ($P \leq 0.05$).

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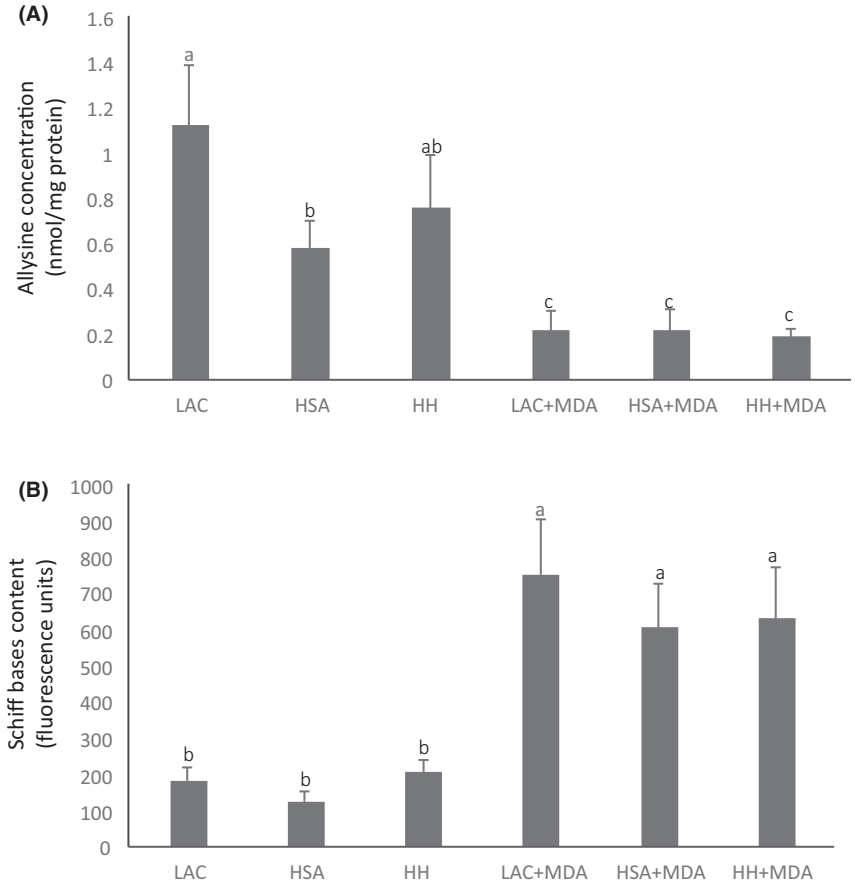

Fig. 4. Concentration of allysine (A) and Schiff bases (B) (means ± standard deviation) in human serum albumin (HSA), human haemoglobin (HH) and β-lactoglobulin (LAC) (5 mg ml^{-1}) after incubation with MDA (0.25 μM) at 37 °C (HSA, HH) and 80 °C (LAC) for 24 h. Means for each experimental group are calculated from analyses applied to three biological replicates and all analyses were technically duplicated. Different letters denote significant differences between means within the same sampling point in ANOVA ($P \leq 0.05$).

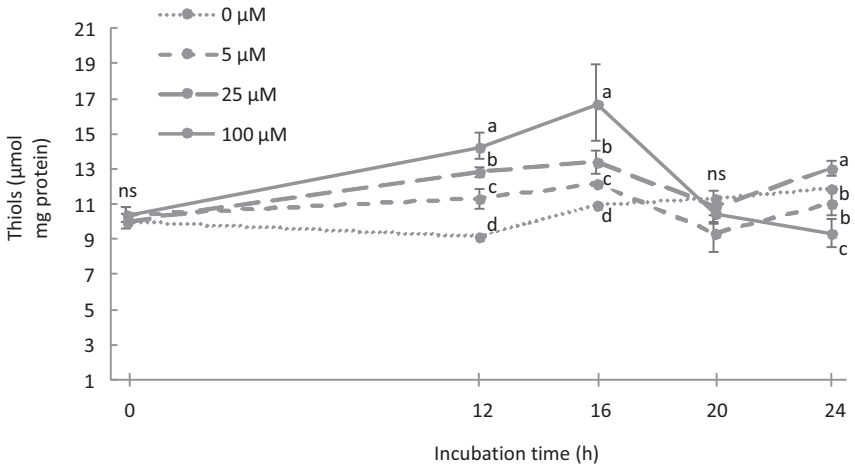

Fig. 5. Concentration of free thiols (means ± standard deviation) in *Lactobacillus reuteri* PL503 grown in MRS broth with increasing concentrations of malondialdehyde (MDA) during an incubation period for up to 24 h. Means for each experimental group are calculated from analyses applied to three biological replicates and all analyses were technically duplicated. Different letters at the same sampling time denote significant differences between means within the same sampling point in ANOVA ($P \leq 0.05$).

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the counts remained stable during the entire experimental assay (37 °C/24 h). This finding reflects the ability of *L. reuteri* to activate mechanisms to neutralize the potential harmful effects of the sublethal concentrations of this lipid peroxidation product. In the present study, these mechanisms were firstly assessed by the analysis of the relative transcription of stress-related genes.

uspA *gene*

Considering the role of the *uspA* gene in the defence against DNA-damaging agents (Kvint *et al*., 2003), the upregulation of such gene as a response to the challenge of MDA was expected. MDA is known to form adducts with assorted biomolecules, including the DNA (Esterbauer *et al*., 1993). Marnett (1999) reported that MDA reacts with DNA to form adducts to deoxyguanosine and deoxyadenosine. However, the major adduct to DNA is a pyrimidopurinone called M1G, which has been also identified in human liver, white blood cells, pancreas and breast tissues, and is considered a significant contributor to cancer linked to dietary factors (Niedernhofer *et al*., 2003). It is hence, relevant to find out whether colonic microbiota is able to detoxify and/or counteract the noxious effects of MDA. In bacteria, M1G has been also found to lead to mutagenesis, which is repaired by the nucleotide excision repair pathway (Marnett, 1999). The threat of such mutagenesis could have caused an upregulation of the *uspA* gene, as shown in Fig. 1A. These results are, however, divergent to those reported by Oberg *et al*. (2015) who observed a significant inhibition of the *uspA* gene expression in *Bifidobacterium longum* exposed to a hydroxyl radical generating system. Conversely, an ATP-dependent metallo-protease was found to be upregulated to likely protect membrane proteins against radical-mediated oxidative damage. The specificity of the genetic responses of a given bacterium challenged with different pro-oxidant threats seems plausible since the damage caused by MDA via adduct formation to biomolecules (including proteins and DNA) is fairly different from the severe oxidative damage caused by ROS.

dhaT *gene*

While the *uspA* gene encodes an assorted collection of proteins, the expression of the *dhaT* gene leads to the synthesis of a single protein with definite function, the 1,3 propanediol oxidoreductase (1,3-PDO; Schaefer *et al*, 2010). This enzyme plays a relevant role in stress situations involving energetic demand since 1,3-PDO facilitates the main carbohydrate fermentation pathway (6-phosphogluconate/phosphoketolase; 6-PG/PK) through the production of NAD^+ (required for glucose fermentation) from NADH in the conversion of 3-hydroxypropionaldehyde (3-HPA) (its substrate) into 1,3 propanediol (1,3-PD) under anaerobic conditions. Additionally, 3-HPA, also known as reuterin, is known to be profusely excreted to the surrounding environment under stress situations, imparting strong antimicrobial properties (Schaefer *et al*, 2010).

The underlying mechanisms by which *L. reuteri* may try to protect against MDA-induced biological damage through the activation of the 3-HPA pathway should be a matter of thoroughly analysis. The 3-HPA pathway, as previously reported, requires the presence of glycerol, commonly added as growth promoter in *Lactobacillus* cultures (Talarico *et al*, 1988). Since *L. reuteri* from the present experiment had no access to glycerol, and the production of 3-HPA, preferential target of 1,3-PDO, may not be present in the media, the purpose of the upregulation of the gene encoding the 1,3-PDO remains indefinite. It is clear, however, that such enzyme may be implicated in a protection mechanism against MDA-induced oxidative stress that reasonably involves the $NADH/NAD^+$ redox pair and that 3-HPA may not be the unique substrate for 1,3-PDO. In a previous study (Arcanjo *et al*., 2019), in which *L. reuteri* was challenged with H_2O_2 (0.5 mM) in the absence of glycerol, a similar effect on the expression of the *dhaT* gene was observed. The authors hypothesized whether the NAD+-dependent activity of the 1,3-PDO may be able to detoxify H_2O_2 in the presence of NADH in accordance to the pathway proposed in Fig. 6. Interestingly, *Lactobacillus* spp. have been also found to be able to generate H_2O_2 and other ROS via implication of NAD(P)H oxidoreductases (Hertzberger *et al*, 2014). The incubation of *L. reuteri* PL503 in the presence of MDA led to increase the production ROS as shown in Fig. 2. The analysis of the bacterium with flow cytometry showed that increasing concentrations of MDA led to accumulative collection of cells with significant generation of ROS. These results originally prove that MDA induces the formation of diverse radical species in *L. reuteri* PL503 plausibly via production of H_2O_2, the most common source of hydroxyl radical in biological systems (Davies, 2005). The production of H_2O_2 by *Lactobacillus* spp., already reported in literature, seems to be promoted by the presence of electron acceptors such as O_2 and fructose (Mane, 2016). MDA, as a potent electron acceptor, may also have such effect on *L. reuteri* PL503 supporting the connection between the lipid peroxidation product, the impairment of the redox status of the cell, the production of H_2O_2 and the upregulation of the gene encoding a NADH-dependent oxidoreductase (Fig. 6). However, the underlying mechanism by which MDA would promote ROS formation in *L. reuteri* requires further elucidation. Although *Lactobacillus* spp. can produce antimicrobial

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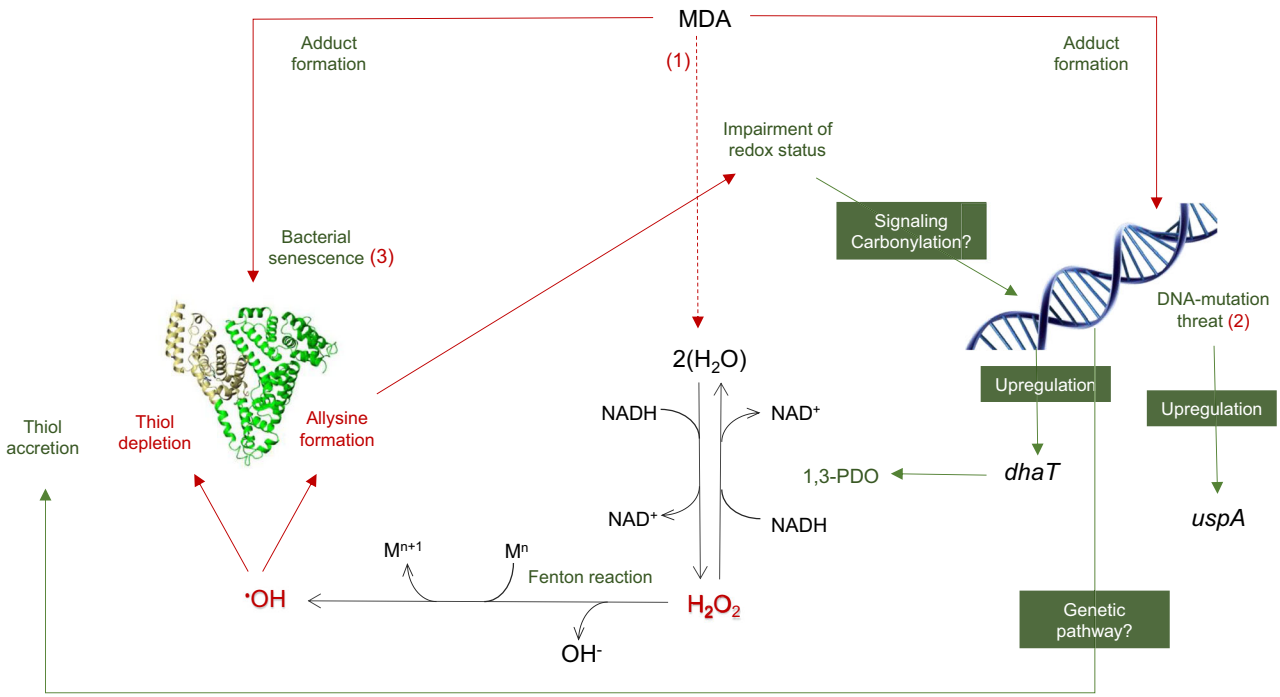

(1) MDA-treated bacteria were found to have higher concentration of hydrogen peroxide and ROS. While mechanisms are yet to be elucidated, the formation of hydrogen peroxide by a NADH-dependent mechanism is proposed in accordance to Hertzberger et al. (2014).

(2) MDA is known to adduct to DNA bases and induce mutation in bacteria (Draper et al. 1986).

(3) MDA-mediated protein carbonylation has been found to cause impairment of biological processes and senescence in bacteria (Ezraty et al. 2017).

Fig. 6. General scheme of the proposed mechanisms whereby malondialdehyde (MDA) damages biomolecules in *Lactobacillus reuteri* PL503 (red lines) and mechanism by which the bacterium may protect against MDA-induced oxidative stress (green lines).

peptides, bacteriocins and several organic compounds, releasing H_2O_2 seems to be central for antimicrobial and restorative processes (Singh *et al*, 2018). This has been also highlighted as a relevant probiotic mechanism as ROS production by *Lactobacillus* spp. has been shown to promote epithelial restitution during colitis and alleviate inflammation in human mucosa (Singh *et al*, 2018). Finally, physiological production of radical species induced by pro-oxidative compounds such as MDA can contribute cells to minimize oxidative stress. This mechanism may involve radical species acting as signalling molecules or inducing subtle modifications in proteins that may, in turn, act as signalling molecules that could eventually enhance endogenous antioxidant mechanisms in the bacterium and in the host (Martín and Suarez, 2010). While the H_2O_2 production in *Lactobacillus* spp. is well documented, the mechanisms are not definite though it may involve the conjunction of NADPH and oxidoreductase enzymes (proposed mechanism in Fig. 6). The overexpression of the *dhaT* gene, alleged to protect against H_2O_2-induced oxidative stress, may respond to the necessity of the bacterium to counteract the potential damage that such pro-oxidant species may exert in its own biomolecules.

The pathogenesis of particular chemical species depends on their ability to establish molecular interactions with biomolecules from the host and as a result, impair biological processes. MDA is known to exert harmful effects by adducting biomolecules such as DNA and proteins (Bartsch and Nair, 2005). While the adducts with DNA and the corresponding MDA-induced mutations have been known for some time in bacteria (Draper *et al*., 1986), the impact of MDA on proteins of biological relevance is not so well understood. Protein oxidation is not only mediated by ROS, as oxidizing lipids and final lipid oxidation products have been also identified as potential initiators of oxidative reactions in proteins (Davies, 2005). The damage caused to proteins in pro-oxidative environments has been emphasized as one of the most salient causes of ageing and disease in humans (Davies, 2005; Estévez and Luna, 2017). Protein oxidation also has devastating effects on the structure and functionality of bacteria which may even lead to bacterial senescence and cell death (Ezraty *et al*., 2017). Interestingly, the oxidative damage to proteins plays a role in the bacterial response to oxidative stress (Ezraty *et al*., 2017) as proteins are activated by oxidative means to trigger specific antioxidant mechanisms. In

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order to provide further insight into the effects of MDA on *L. reuteri*, protein oxidation was assessed through the quantification of a specific protein carbonyl (allysine) and the formation of Schiff bases in bacterial proteins. Free thiols, as relevant redox-active moieties in proteins, were also quantified.

Protein oxidation

Allysine is the most abundant protein carbonyl in biological systems and typically used as indicator of the oxidative damage to proteins (Estévez, 2011). In tissues from mammals, a concentration of 1 nmol carbonyls per mg protein has been reported as physiological while significant increases are commonly reflecting oxidative stress conditions (Akagawa *et al.*, 2002). This is, to our knowledge, the first time that such specific protein oxidation product is quantified in cultured bacteria as marker of oxidative stress. Measuring protein carbonylation using the routine spectrophotometric DNPH method, Ballesteros *et al.* (2001) proposed this expression of the oxidative damage to proteins as a reflection of bacterial senescence as oxidized proteins accumulate in non-proliferating bacteria. This is consistent with the evolution of allysine in CONTROL group of *L. reuteri* PL503. The present results show that allysine, formed in bacteria, as in eukaryotes, may be used as a reliable indicator of protein oxidation. Allysine is formed in proteins as an outcome of oxidative deamination of the ε-amino group in lysine residues and that oxidation pathway can be initiated by i) radical species (i.e. hydroxyl radical) (Utrera and Estevez, 2013) or by dicarbonyls from the Maillard reaction (i.e. glyoxal/methylglyoxal) (Akagawa *et al.*, 2002). MDA was found to promote the formation of allysine in *L. reuteri* PL503 and we performed additional analyses to find out the underlying mechanism of such oxidative damage. It is known that MDA reacts with ε-amino group in lysine residues but the formation of allysine as an outcome of such reaction is not described in the literature. The additional assay carried out with human and bovine proteins (Fig. 4A,B) confirmed that MDA is not able to induce the oxidative deamination of alkaline amino acids via the Maillard mechanism previously reported. Instead, the reaction of MDA with such residues is known to yield Schiff bases and stable protein crosslinks (Requena *et al.*, 1997; Estévez *et al.*, 2019). While the formation of such fluorescent structures was the most likely fate of MDA residues in isolated human and bovine proteins, the evolution of Schiff bases in bacteria was irregular and considerably low as compared to other studies carried out in animal proteins (Utrera and Estevez, 2013). It is then reasonable to hypothesize that the significant decrease in MDA, previously stated (Fig. 3A), may respond to reactions with other biomolecules, including DNA, which would explain the fast and dose-dependent overexpression of the *uspA* gene, involved in the defence against DNA-damaging agents. These results suggest that MDA promoted allysine formation in *L. reuteri* PL503 through mechanisms that likely involve a ROS-mediated pathway. This hypothesis is supported by the increase of ROS detected by flow cytometry in the bacterium treated with MDA (Fig. 2). This mechanism may involve the previous formation of H_2O_2 and its subsequent decomposition through the Fenton reaction into hydroxyl radicals (Proposed mechanism depicted in Fig. 6). The radical-mediated oxidative deamination of lysyl residues is, so far, the most plausible mechanism behind the formation of allysine in *L. reuteri* PL503 incubated with MDA.

The biological significance of protein carbonylation should be another issue of discussion. As an irreversible modification in proteins in a pro-oxidative environment, protein carbonylation is typically regarded as a reaction of negative biological consequences. Carbonylated proteins can be dysfunctional and may be tagged to removal as their accumulation causes impaired homeostasis that leads to chronic dysfunction and apoptosis (Shacter, 2000). On the other hand, carbonylated proteins may also act as signalling molecules, which may trigger specific pathways, aimed to preserve homeostasis control senescence (Shacter, 2000). Both circumstances may be applied to the present experiment. The initial increase in protein carbonyls in the MDA-treated bacterium (16 h) was followed by a notable decrease of allysine in *L. reuteri* PL503 exposed to the highest MDA concentration. In these samples, at concentrations around 3 nmol allysine mg^{-1} protein, such bacterium may have activated the *dhaT* gene, clearly noticeable in the following sampling times. The NADH-dependent oxidoreductase decoded by this gene may have contributed to detoxify pro-oxidant species such as H_2O_2 and hence, inhibiting the enduring carbonylation observed in *L. reuteri* treated with lower doses of MDA (Fig. 6). The allysine decline by the end of the assay in *L. reuteri* PL503 treated with 100 μM MDA can only respond to the removal of carbonylated proteins through a mechanism (not identified in the present study) that could have been likely activated along with the *dhaT* pathway. It is worth noting that these mechanisms were not present in the bacterium incubated with 5 and 25 μM as allysine concentrations higher than 3 nmol allysine mg^{-1} protein were only reached at the end of the assay and regrettably the events that could have happened in hypothetical further sampling times are ignored. The hypothesis that the *dhaT* gene could have been activated by H_2O_2 and/or the effect of the former

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on protein carbonylation is supported by previous considerations made by Ezraty *et al*. (2017) and Arcanjo *et al*. (2019). The latter authors, in particular, observed how the accumulation of carbonyls in *L. reuteri* PL503, as a irreversible modification in oxidized proteins, triggered the upregulation of the *dhaT* gene in the presence of H_2O_2 and resveratrol.

Free thiols

Sulfur-containing amino acids such as cysteine (Cys) and methionine (Met) are particularly sensitive to oxidation and thiol depletion is a typical feature in oxidized proteins. While the oxidation of thiols in the active site of enzymes may lead to dysfunction, irrelevant sulfur-containing amino acids are known to act as endogenous antioxidants by offering a sacrificial loss to ROS and protecting other amino acids with relevant biological significance (Estévez *et al*, 2020). This double role of thiols was investigated in the present experiment. A paired balance between thiol oxidation and repair/*de novo* synthesis of proteins occurred during the first 12 h in CONTROL bacteria owing to the absence of oxidative stress. During the second half of the assay, the timely coincidence of thiol accretion with the increase of carbonylation in CONTROL samples may respond to a physiological strategy to keep a balanced redox status in a senescent cell. The pro-oxidant changes induced by MDA in bacteria, including the formation of protein carbonyls, plausibly triggered the accretion of thiol groups by *de novo* synthesis of sulfur-containing proteins/peptides and as a result, protect the bacterium against potential pro-oxidant threats (Fig. 5). Thiols are typically regarded as elements of antioxidant protection in eukaryotes and also, in lactic acid bacteria (Schaefer *et al*., 2010; Xiao *et al*., 2011). Yet, the underlying molecular mechanisms behind the synthesis of thiol-containing species remain indefinite and needs further elucidation.

The depletion of thiols observed in the following sampling points may respond to two divergent circumstances depending on the concentration of MDA. In cultures with 100 μM MDA, the control of the oxidative threat through a timely genetic response (overexpression of the *uspA* and particularly the *dhaT* genes, among others) allowed lowering the amount of protein carbonyls to physiological levels and hence, the accretion of thiols may not be required anymore and was also lowered at basal levels. In the groups of *L. reuteri* PL503 treated with 5 and 25 μM, an insufficient genetic response did not avoid MDA-induced ROS generation and the subsequent protein carbonylation, and hence, the drop in thiols may respond to the consumption of such moieties in the sacrificial loss aforementioned. In probiotic bacteria such as *L. reuteri*, this mechanism may contribute to their capability to counteract ROS and hence, defend themselves and the host against oxidative stress.

Enclosing, the present study provides original insight into the molecular and genetic responses of *L. reuteri* PL503 to the toxic effects of MDA, one of the most common lipid peroxidation products. This bacterium is able to detoxify MDA and hence, exert a potential health benefit in the gastrointestinal tract. Contributing to identify some of the underlying genetic and biochemical mechanisms facilitate the development targeted prophylactic and treatment strategies involving this and other probiotic bacteria. While the *uspA* and *dhaT* genes have been found to be strongly affected by MDA and likely play a relevant role in the response of the probiotic bacteria to oxidative stress, other genes and metabolic pathways may have been affected. Therefore, further genomic studies are required to unveil which other genes and metabolic routes may be involved in the responses of probiotic bacteria to the oxidative stress induced by MDA. The health benefits of dietary supplementation may be proven in further clinical studies.

Experimental procedures

Chemicals and raw material

All chemicals and reagents used in this study were of American Chemical Society (ACS) analytical grade and purchased from Sigma Chemicals (Sigma-Aldrich, Germany), Scharlab S.L. (Spain), Pronadisa (Conda Laboratory, Spain), Applied Biosystems (USA), Epicentre (USA) and Acros Organics (Spain). *L. reuteri* PL503 isolated from pig faeces was previously identified by 16S rRNA gene sequencing (Ruiz-Moyano *et al*., 2008).

Experimental setting

Stock cultures of *L. reuteri* PL503 were stored at −80 °C in Man, Rogosa and Sharpe (MRS) broth supplied with glycerol to a final concentration of 20% (v/v). Before experimental use, *L. reuteri* PL503 was subcultured twice under microaerophilic conditions at 37 °C for 24 h in MRS broth supplemented with 0.5% of diluted acetic acid (10%, v/v). Four experimental groups were considered depending on the concentration of MDA added: CONTROL (*L. reuteri*), 5 μM (*L. reuteri* + 5 μM MDA), 25 μM (*L. reuteri* + 25 μM MDA) and 100 μM (*L. reuteri* + 100 μM MDA). Three replicates were carried out for each treatment. Experimental tubes were inoculated with 100 μl of the last overnight culture of *L. reuteri* PL503 in MRS broth and incubated at 37 °C for up to 24 h in microaerophilic conditions. The bacterial counts ranged from 9.6 to 10 log cfu ml^{-1} during the entire assay and no significant changes were observed

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between experimental groups. Samples of the cultures were collected in four times (12, 16, 20 and 24 h) from the inoculation. For further protein analyses, culture medium was removed by washing with a phosphate-buffered saline (PBS, pH 7.4) solution twice. For counting of viable cells, 100 μl of *L. reuteri* PL503 was inoculated on MRS agar at the same sampling time and conditions as the experimental tubes.

Gene expression studies

Extraction of RNA. After each incubation time, 1 ml samples of each treatment were frozen and stored at –80 °C. RNA was extracted using the MasterPure™ RNA purification kit (Epicentre), which includes DNase treatment, as described by the manufacturer. Pure RNA was eluted in 35 μl Tris (200 mM) EDTA (20 mM) buffer (pH 8) and kept at –80 °C until required. The RNA concentration (ng μl^{-1}) and purity (A_{260}/A_{280} ratio) were spectrophotometrically determined using a 1.5 μl aliquot on the Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA).

cDNA synthesis

cDNA was synthesized using about 500 ng of total RNA following the instructions of the PrimeScript™ RT Reagent kit (Takara Bio Inc., Kusatsu, Japan). cDNA was stored at −20 °C until further use.

Real-time PCR analysis of gene expression

The *uspA* and *dhaT* genes were selected for relative expression studies using real-time PCR (qPCR). The 16S gene was used as reference gene. The qPCR assays were performed and monitored in a ViiA™ 7 Real-Time system (Applied Biosystems, Foster City, CA, USA) using MicroAmp optical 96-well reaction plates, sealed with optical adhesive covers (Applied Biosystems). qPCR results were analysed using the Software ViiA™ 7 RUO v1.2.4. (Applied Biosystems). The SYBR Green technology was used. The reaction mixture (final volume, 12.5 μl), contained 2.5 μl of cDNA, 6.25 μl of SYBR® Premix Ex Taq™ (Takara Bio Inc.), 0.625 μl of ROX™ Reference Dye (Takara Bio Inc.) and 300 mM of each primer pair. Some primers were specifically develop for this study such as uspALr-F1 (CTTGGGTAGCGTTCACCATT) and uspALr-R1 (TGA AAAAGCGGTTGACACTG) for the *uspA* gene (annealing temperature: 60 °C) and Lr16S_F (CCGC TTAAACTCTGTTGTTG) and Lr16S_R (CGTGACT TTCTGGTTGGATA) for the 16S gene (annealing temperature: 55 °C). The primers LS67 (TGACTGGATCC-TAATTTGGTCCTGGTGTTATTGC) and LS68 (TGACTGAATTCTTCCGGATCTTAGGGTTAGG) were designed in accordance to Schaefer et al. [50] for the *dhaT* gene (annealing temperature: 60 °C).

The qPCR programme consisted of initial denaturation step at 95 °C for 10 min at 95 °C; 40 cycles at a denaturation temperature of 95 °C for 15 s and annealing/extension temperatures of 55 °C and 60 °C for the 16S and target genes, respectively, during 30 s. After the final qPCR cycle, a melting curve was included by heating the product from 60 to 99 °C and continuous measurement of the fluorescence was performed to verify the qPCR products. All samples were analysed in triplicate, including control sample consisting of adding sterile ultrapure water instead of cDNA. The expression ratio was calculated using the $2^{-\Delta\Delta C_T}$ method reported by Livak and Schmittgen (2001). The calibrator sample corresponded to the value of the expression of the experimental group CONTROL at each sampling time.

Study of in vitro reactivity of MDA with proteins. In order to evaluate the ability of MDA to induce carbonylation in proteins, three proteins, namely, human serum albumin (HSA), human haemoglobin (HH) and bovine β-lactoglobulin (LAC) (5 mg ml^{-1}, final concentration) were dissolved in 100 mM phosphate buffer pH 6.5 and incubated separately with MDA (0.25 μM) at 37 °C (HSA, HH) and 80 °C (LAC in an oven at constant stirring for 24 h. Proteins were selected based on their previously reported susceptibility to oxidative damage (Luna and Estévez, 2018, 2019). MDA concentration was set at preliminary tests aimed to find sublethal concentrations within the range found in colonic digests. Samples were taken at 24 h for the quantification of allysine and Schiff bases. The preparation of six experimental units corresponding to the reaction units (HSA-MDA, HH-MDA and LAC-MDA) and the corresponding controls (proteins without MDA) were replicated three times in corresponding independent assay and all analyses were repeated three times in each same experimental unit (9 measurements per analysis and per treatment to calculate means and standard deviations).

Analytical procedures

ROS generation by flow cytometry analyses. Flow cytometry detection of ROS in *L. reuteri* PL503 was performed as determined using previous published protocols (Ortega-Ferrusola *et al.*, 2017; Peña *et al.*, 2018). In brief, *L. reuteri* PL503 (1×10^6) was extended in 1 ml of PBS and stained with CellRox Deep Red (ThermoFisher, Waltham, MA, USA; 5 μM; excitation and emission wavelengths, 644 and 645 nm respectively) for detection of the bacterium producing

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ROS, and Hoechst 33342 (0.5 μM) (excitation and emission wavelengths, 345 nm and 488 nm respectively; Sigma, Steinheim, Germany) to identify the bacterium and remove debris from the analysis. After thorough mixing, the cell suspension was incubated at room temperature in the dark for 25 min, washed in PBS and the samples were immediately run on the flow cytometer. Flow cytometry analyses were conducted using a Cytoflex® flow cytometer (Beckman Coulter, Brea, CA, USA) equipped with violet, blue and red lasers. The instrument was calibrated daily using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment, however, due to emission and excitation characteristics of the combination of probes used, spectral overlap was negligible. Files were exported as FCS files and analysed using FlowjoV 10.5.3 Software for Mac OS (Ashland, OR, USA). Unstained, single-stained, and Fluorescence Minus One (FMO) controls were used to determine compensations and positive and negative events, as well as to set regions of interest.

Synthesis of allysine standard compound. N-Acetyl-L-AAS (allysine) was synthesized from Nα-acetyl-L-lysine using lysyl oxidase activity from egg shell membrane following the procedure described by Akagawa *et al.* (2002). Briefly, 10 mM Nα-acetyl-L-lysine was incubated under constant stirring with 5 g egg shell membrane in 50 ml of 20 mM sodium phosphate buffer, pH 9.0 at 37 °C for 24 h. The egg shell membrane was then removed by centrifugation and the pH of the solution adjusted to 6.0 using 1 M HCl. The resulting aldehydes were reductively aminated with 3 mmol ABA (4-aminobenzoic acid) in the presence of 4.5 mmol sodium cyanoborohydride ($NaBH_3CN$) at 37 °C for 2 h under stirring. Then, ABA derivatives were hydrolysed by 50 ml of 12 M HCl at 110 °C for 10 h. The hydrolysates were evaporated at 40 °C *in vacuo* to dryness. The resulting allysine-ABA was purified by using silica gel column chromatography and ethyl acetate/acetic acid/water (20:2:1, v/v/v) as elution solvent. The purity of the resulting solution and authenticity of the standard compounds obtained following the aforementioned procedures were checked by using MS and ^{1}H NMR (Estévez *et al.*, 2009).

Quantification of allysine

Five hundred microlitres of culture were dispensed in 2 ml microtubes and treated with cold (4 °C) 10% Trichloroacetic acid (TCA) solution. Each microtube was vortexed and then subjected to centrifugation at 600 *g* for 5 min at 4 °C. The supernatants were removed and the pellets were incubated with the following freshly prepared solutions: 0.5 ml 250 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 6.0 containing 1 mM diethylenetriaminepentaacetic acid (DTPA), 0.5 ml 50 mM ABA in 250 mM MES buffer pH 6.0 and 0.25 ml 100 mM $NaBH_3CN$ in 250 mM MES buffer pH 6.0. The tubes were vortexed and then incubated in water bath at 37 °C for 90 min. The samples were stirred every 15 min. After derivatization, samples were treated with a cold (4 °C) 50% TCA solution and centrifuged at 1200 *g* for 10 min. The pellets were then washed twice with 10% TCA and diethyl ether-ethanol (1:1). Finally, the pellet was treated with 6N HCl and kept in an oven at 110 °C for 18 h until completion of hydrolysis. The hydrolysates were dried *in vacuo* in a centrifugal evaporator. The generated residue was reconstituted with 200 μl of milliQ water and then filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 μm pore size, Pall Corporation, USA) for HPLC analysis.

A Shimadzu 'Prominence' HPLC apparatus (Shimadzu Corporation, Japan), equipped with a quaternary solvent delivery system (LC-20AD), a DGU-20AS online degasser, a SIL-20A auto-sampler, a RF-10A XL fluorescence detector, and a CBM-20A system controller, was used. An aliquot (1 μl) from the reconstituted protein hydrolysates was injected and analysed in the above-mentioned HPLC equipment. AAS- ABA was eluted in a Cosmosil $5C_{18}$-AR-II RP-HPLC column (5 μm, 150 × 4.6 mm) equipped with a guard column (10 × 4.6 mm) packed with the same material. The flow rate was kept at 1 ml min^{-1} and the temperature of the column was maintained constant at 30 °C. The eluate was monitored with excitation and emission wavelengths set at 283 and 350 nm respectively. Standards (0.1 μl) were run and analysed under the same conditions. Identification of both derivatized semialdehydes in the FLD chromatograms was carried out by comparing their retention times with those from the standard compounds. The peak corresponding to allysine-ABA was manually integrated from FLD chromatograms and the resulting areas plotted against an ABA standard curve with known concentrations that ranged from 0.1 to 0.5 mMn (Utrera *et al.*, 2011). Results were expressed as nmol of allysine per mg of protein.

Analysis of Schiff bases

The formation of Schiff bases (SB) was assessed using a LS-55 PerkinElmer fluorescence spectrometer (PerkinElmer, Waltham, MA, USA). Prior to the analysis, reaction mixtures were diluted (1:20) with 8 M urea in 100 mM sodium phosphate buffer, pH 7. SB was excited at 350 nm and the emitted fluorescence recorded at 450 nm. The excitation and emission slit widths were set at 10 nm and the speed of data collection while

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scanning was of 500 nm per min. The height of the peaks corresponding to SB spectra was recorded. After taking into consideration the applied dilutions, the results were expressed as fluorescence units.

Analysis of protein thiols

To avoid possible contamination with thiols from the medium, 250 μl of each *L. reuteri* PL503 culture were washed twice with PBS and with ethanol:ethyl acetate (1:1). The pellet was resuspended in 250 μl of guanidine hydrochloride and added to the cuvette in a final volume of 1250 μl of guanidine hydrochloride. Absorbance was measured at 324 nm, pre and post addition of 250 μl of 4 DPS (4,4′-Dipyridyl disulfide) in 12 mM HCl. Results were expressed as μmol of free thiol groups per mg of protein.

Analysis of thiobarbituric-reactive substances

Malondialdehyde and other thiobarbituric-reactive substances (TBARS) was measured in all samples from 200 μl of each *L. reuteri* PL503 culture, adding 500 μl thiobarbituric acid (0.02 M) and 500 μl trichloroacetic acid (10%), incubating during 20 min at 90 °C. After cooling, a 5 min centrifugation at 600 *g* was made and the supernatant was measured at 532 nm. Results are expressed as mg TBARS per L of sample.

Statistical analysis

Data from the analysis (n = 3) were collected and subjected to statistical analysis. The effect of different concentrations of MDA and incubation times on the chemical measurements, analyses of variance (ANOVA) was applied [SPSS v. 15.5, IBM (Endicott, NY, USA)]. The effect of MDA on the gene expression ($\Delta\Delta C_T$ values) was analysed using paired Students' *t*-tests (SPSS v. 15.5). The statistical significance was set at $P \leq 0.05$.

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Author contributions

M.E. conceived the project and finalized the manuscript. M.E., M.J.A and A.R. designed the experiments and analysed the data. P.P. collected the samples, conducted the PCR and allysine analyses and drafted the manuscript. F.J.P. conducted the cell cytometry analysis. All authors contributed to interpreting the data, reviewed and approved the submission of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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4. II: Malondialdehyde Interferes with the Formation and Detection of Primary Carbonyls in Oxidized Proteins

El malondialdehído interfiere en la formación y detección de carbonilos primarios en proteínas oxidadas.

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Research Paper

Malondialdehyde interferes with the formation and detection of primary carbonyls in oxidized proteins

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ABSTRACT

Carbonylation is one of the most remarkable expressions of the oxidative damage to proteins and the DNPH method the most common procedure to assess protein oxidation in biological samples. The present study was elicited by two hypotheses: i) is malondialdehyde, as a reactive dicarbonyl, able to induce the formation of allysine through a Maillard-type reaction? and ii) to which extent does the attachment of MDA to proteins interfere in the assessment of protein carbonyls using the DNPH method? Human serum albumin (HSA), human hemoglobin (HEM) and β-lactoglobulin (LAC) (5 mg/mL) were incubated with MDA (0.25 mM) for 24 h at 37 °C (HSA and HEM) or 80 °C (LAC). Results showed that MDA was unable to induce oxidative deamination of lysine residues and instead, formed stable and fluorescent adducts with proteins. Such adducts were tagged by the DNPH method, accounting for most of the protein hydrazones quantified. This interfering effect was observed in a wide range of MDA concentrations (0.05–1 mM). Being aware of its limitations, protein scientists should accurately interpret results from the DNPH method, and apply, when required, other methodologies such as chromatographic methods to detect specific primary oxidation products such as allysine.

1. Introduction

The oxidation of proteins has become a topic of undeniable interest among biochemists given the role of the oxidative damage to proteins in cell function, disease and aging [1–3]. As a posttranslational modification in proteins, oxidation can be part of a precise physiological mechanism (i.e. cellular signaling) or the outcome of uncontrolled oxidative stress. The removal of oxidized proteins responds to a strategy to avoid protein dysfunction and altered physiological processes. Yet, when proteins are severely damaged, they accumulate in cells leading to chronic disorders [2]. Food scientists and nutritionists have also reported the implications of protein oxidation in food systems and they include i) impaired functionality and digestibility ii) altered sensory properties and iii) potential safety concerns as a result of the intake of oxidized proteins and amino acids [4,5].

Carbonylation is one of the most remarkable expressions of the oxidative damage to proteins [6]. The on-site formation of carbonyls in proteins (~primary protein carbonyls) typically occurs as the result of the attack of reactive oxygen species (ROS) to the ε-amino group of susceptible amino acids (lysine, arginine and proline). However, this oxidative deamination mechanism is also triggered by α-dicarbonyls such as glyoxal (GO) and methylglyoxal (MGO), formed from the degradation of reducing sugars [7] (Fig. 1). This Maillard-mediated mechanism has been found to occur in pathological disorders (i.e. diabetes) involving high concentration of circulating glucose [7] and also in food systems in which reducing sugars and their oxidation products have been found to react with ε-amino group of alkaline amino acids in proteins [8,9]. Regardless of the underlying mechanism (ROS-mediated or Maillard-mediated), the oxidative deamination of lysine, one of the most abundant amino acids in proteins, leads to the formation of allysine, a primary protein carbonyl and reliable marker of oxidative stress and disease [6]. Proteins can also be carbonylated by the addition of pre-formed carbonyl groups such as those generated from lipid oxidation (~secondary protein carbonyls) [10]. Malondialdehyde (MDA) and 4-hydroxy-non-2-enal (4-HNE), among others, form covalent linkages with proteins by reacting, precisely, with ε-amino groups from protein-bound lysines. Some of these adducts (Fig. 2) have been linked to assorted pathological conditions [11,12]. It is, however, ignored,

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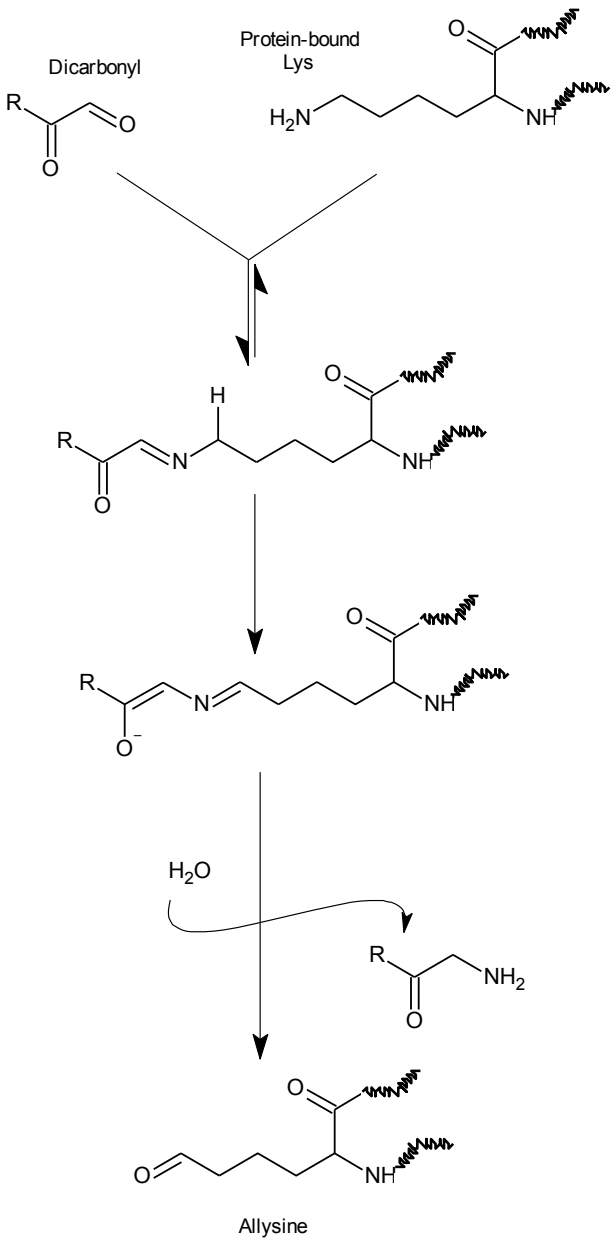

Fig. 1. Formation pathway of allysine in the presence of an α-dicarbonyl in accordance to the Maillard-mediated mechanism proposed by Akagawa et al. (2005).

whether MDA and other dicarbonyls from lipid oxidation are able to replicate the Maillard-mediated mechanism of GO and MGO and hence, induce on-site carbonylation of alkaline amino acids (e.g. formation of protein-bound allysine).

While all these mechanisms may occur simultaneously, each of them responds to completely different reaction conditions and their implications and consequences for the biological system, whether this is a living organism or food system, may also be different. In light of this complex chemistry, specific methodological approaches are needed to identify which particular mechanism is responsible for the carbonylation of proteins in a given biological sample. Yet, the most common procedure for quantifying protein carbonyls in biological systems does not provide such relevant information. This routine method involves tagging all carbonyl moieties with the dinitrophenylhydrazine (DNPH) reagent [13]. The DNPH reacts with all protein carbonyls, regardless of their formation pathway, and furthermore, lipid-derived carbonyls such as MDA are also tagged. In fact, routine methods for the quantification of MDA in plasma and other biological samples involve using DNPH as a derivatization agent [14].

The hypotheses that elicited the present study were: i) is MDA, as a reactive dicarbonyl, able to induce the formation of allysine through a Maillard-type reaction (Fig. 1) and ii) to which extent does the attachment of MDA to proteins interfere in the assessment of protein carbonyls using the DNPH method?

2. Material and methods

2.1. Chemicals

All chemicals, reagents and proteins used for the present work were purchased from Panreac (Panreac Química, S. A., Barcelona, Spain), and Sigma Chemicals (Sigma- Aldrich, Steinheim, Germany). Water used was purified by passage through a Milli-Q system (Millipore Corp., Bedford, MA). The molarities of all reactants refer to the final concentration in the reaction mixture.

2.2. Synthesis of allysine

N-Acetyl-L-AAS (allysine) was synthesized from *N*-acetyl-L-lysine using lysyl oxidase activity from egg shell membrane following the procedure described by Akagawa et al. [15]. Briefly, 10 mM *N*-acetyl-L-lysine was incubated at constant stirring with 5 g egg shell membrane in 50 mL of 20 mM sodium phosphate buffer, pH 9.0 at 37 °C for 24 h. The egg shell membrane was then removed by centrifugation and the pH of the solution adjusted to 6.0 using 1 M HCl. The resulting aldehydes were reductively aminated with 3 mmol p-amino-benzoic acid (PABA) in the presence of 4.5 mmol sodium cyanoborohydride ($NaBH_3CN$) at 37 °C for 2 h with stirring. Then, PABA derivatives were hydrolyzed by 50 mL of 12 M HCl at 110 °C for 10 h. The hydrolysates were evaporated at 40 °C *in vacuo* to dryness. The resulting allysine-PABA was purified by using silica gel column chromatography and ethyl acetate/acetic acid/water (20:2:1, v/v/v) as elution solvent. The purity of the resulting solution and authenticity of the standard compounds obtained following the aforementioned procedures were checked by using MS and ^{1}H NMR [16].

2.3. Experimental setting

In order to evaluate the ability of MDA to induce carbonylation in proteins, three proteins, namely, human serum albumin (HSA), human hemoglobin (HEM) and β-lactoglobulin (LAC) (5 mg/mL, final concentration) were dissolved in 100 mM phosphate buffer (pH 6.5) and incubated separately with MDA (0.25 mM) at 37 °C (HSA, HEM) and 80 °C (LAC) in an oven at constant stirring for 24 h. Proteins were selected on the basis of their susceptibility to oxidative damage [9,17]. Samples were taken at fixed incubation times (0, 2, 7 and 24 h) for the quantification of total protein carbonyls by the DNPH method, free amino groups, allysine and Schiff bases. The preparation of six experimental units corresponding to the reaction units (HSA-MDA, HEM-MDA and LAC-MDA) and the corresponding controls (proteins without MDA) were replicated three times in corresponding independent assays and all analyses were repeated three times in each same experimental unit (9 measurements per analysis and per treatment to calculate means and standard deviations).

In order to evaluate the dose effect of MDA on the aforementioned changes, HSA (5 mg/mL, final concentration) was dissolved in 100 mM phosphate buffer (pH 6.5), and incubated with increasing concentrations of MDA (0, 0.05, 0.10, 0.25, 1, 5, 10 and 50 mM) at 37 °C in an oven at constant stirring for 24 h. Samples were taken at 24 h for the quantification of total protein carbonyls by the DNPH method, free amino groups, allysine and Schiff bases. The preparation of eight experimental units corresponding to the each MDA concentration, were

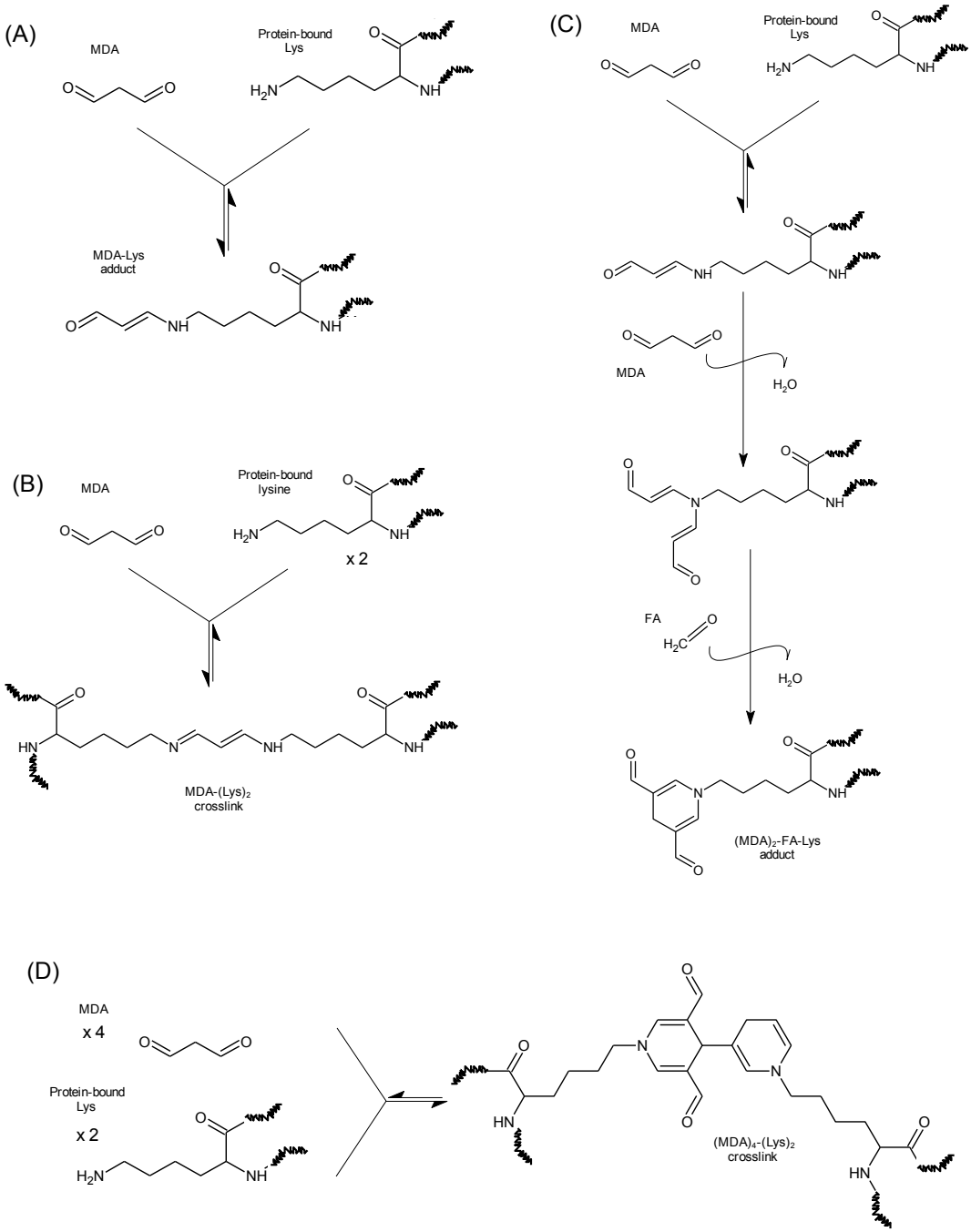

Fig. 2. Formation of adducts between MDA, AA and lysine residues (Adapted from Weiβer et al. [24]; and Nakamura et al. [23]).

replicated three times in corresponding independent assays and all analyses were repeated three times in each same experimental unit (9 measurements per analysis and per treatment to calculate means and standard deviations).

2.4. Protein carbonyls by the DNPH method

Total protein carbonyls were determined by means of the dinitrophenylhydrazine (DNPH) method described by Levine et al. [13] with some modifications. Protein suspensions (200 μL) from each experimental unit were precipitated by the addition of 1 mL of cold 10% trichloroacetic acid (TCA), followed by centrifugation at 4 °C at 600 g for 5 min and the supernatants were discarded. Pellets were treated with 1 mL of a 2 M HCl solution with 0.2% DNPH and incubated at room temperature for 1 h. Proteins were subsequently precipitated with 1 mL of cold 10% TCA, followed by centrifugation at 4 °C, 1200 g for 10 min and washed twice with 1 mL of ethanol:ethyl acetate (1: 1 v/v). The pellets were dissolved in 1.5 mL of 20 mM Na_3PO_4 buffer pH 6.5 added with guanidine hydrochloride to reach 6 M. The amount of carbonyls was expressed in nmoles of protein hydrazones per mg of protein using a molar extinction coefficient of hydrazones (21.0 nM^{-1} cm^{-1}) with absorbance readings at 370 nm.

2.5. Analysis of free amino groups

Free amino groups were quantified as described by Weigele et al. [18] and Strauss & Gibson [19]. Protein suspensions (850 μL) were added to 2.0 mL 0.05 M sodium tetraborate (pH 8.5) in a 4 mL quartz

spectrofluorometer cell. Subsequently, 150 μL of 0.7 mM fluorescamine solution in acetone werte dispensed. The cell was inverted four times, and the resulting fluorescence was measured using 390/485 nm for excitation and emission on a PerkinElmer LS45 Fluorescence spectrometer (Llantrisant, UK). The free amino group concentration was calculated based on a standard curve prepared from lysine diluted in malic acid buffer (pH 5.8). The contribution from malic acid buffer (pH 5.8) was recorded under the same conditions and subtracted from all the samples. The concentration is given as μmol amino groups/mg protein.

2.6. Analysis of Schiff bases by fluorescence spectroscopy

The formation of Schiff bases (SB) was assessed using a LS-55 PerkinElmer fluorescence spectrometer (PerkinElmer, Beaconsfield, UK). Prior to the analysis, reaction mixtures were diluted (1:20) with 8 M urea in 100 mM sodium phosphate buffer, pH 7. SB were excited at 350 nm and the emitted fluorescence recorded at 450 nm. The excitation and emission slit widths were set at 10 nm and the speed of data collection while scanning was of 500 nm per minute. The height of the peaks corresponding to SB spectra was recorded. After taking into consideration the applied dilutions, the results were expressed as arbitrary fluorescence units.

2.7. Analysis of allysine by HPLC

Allysine was identified and quantified upon derivatization with p-amino-benzoic acid (PABA) and subsequent analysis by fluorescent HPLC as reported before by Utrera et al. [20]. Two hundred microliters of protein suspension were dispensed in eppendorf tubes and treated with 1 mL of a cold 10% TCA solution. Each eppendorf was vortexed and then subjected to centrifugation at 2000*g* for 30 min at 4 °C. The supernatant was removed and the pellet was treated with 1 mL of a cold 5% TCA solution. A new centrifugation was performed at 5000 g for 5 min at 4 °C. The supernatant was removed and the pellets were incubated with the following freshly prepared solutions: 0.5 mL 250 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer pH 6.0 containing 1% sodium dodecyl sulfate (SDS) and 1 mM diethylenetriaminepentaacetic acid (DTPA); 0.5 mL 50 mM PABA in 250 mM MES buffer pH 6.0 and 0.25 mL 100 mM $NaBH_3CN$ in 250 mM MES buffer pH 6.0. The eppendorf tubes were vortexed and then incubated in an oven at 37 °C for 90 min. The samples were stirred every 15 min. After derivatization, samples were treated with a cold 50% TCA solution and centrifuged at 5000 g for 10 min. The pellet was then washed twice with 1 mL of cold 10% TCA and 1 mL of diethyl ether-ethanol (1:1). Finally, the pellet was treated with 0.5 mL of 6 N HCl and kept in an oven at 110 °C for 18 h until completion of hydrolysis. Hydrolysates were dried under nitrogen flow while heated in a thermoblock (40 °C). The generated residue was reconstituted with 200 μL of milliQ water and then filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 μm pore size, Pall Corporation, USA) for HPLC analysis.

A Shimadzu 'Prominence' HPLC apparatus (Shimadzu Corporation, Kyoto, Japan), equipped with a quaternary solvent delivery system (LC-20AD), a DGU-20AS on-line degasser, a SIL-20A auto-sampler, a RF-10A XL fluorescence detector (FLD), and a CBM-20A system controller, was used. An aliquot (1 μL) from the reconstituted protein hydrolysates was injected and analyzed in the above mentioned HPLC equipment. Allysine eluted in a Cosmosil 5C18-AR-II RP-HPLC column (5 μm, 150 × 4.6 mm) equipped with a guard column (10 × 4.6 mm) packed with the same material. The flow rate was kept at 1 mL/min and the temperature of the column was maintained at 30 °C. The eluate was monitored with excitation and emission wavelengths set at 283 and 350 nm, respectively. Standard allysine (0.1 μL) was run and analyzed under the same conditions. Identification of derivatized allysine in the FLD chromatograms was carried out by comparing its retention times with that from the standard compound. The peak corresponding to allysine-ABA was manually integrated from FLD chromatograms and the resulting areas plotted against an ABA standard curve with known concentrations that ranged from 0.1 to 0.5 mM. Results are expressed as nmol of allysine per mg of protein.

2.8. Statistical analyses

Data from the analysis of depletion of amino groups and accretion of protein hydrazones, allysine and fluorescent Schiff bases (n = 9 per treatment) in proteins treated with MDA, were collected and subjected to statistical analyses. Normality and homoscedasticity were checked for every data set in order to comply with the ANOVA requirements. In order to assess the effect of MDA addition and incubation time, a two-way analysis of variance (ANOVA) was applied to data (SPSS v. 15.5). In order to assess the effect of MDA dose, a one-way analysis of variance (ANOVA) was applied to data (SPSS v. 15.5). Tukey tests were applied when ANOVA found significant differences among treatments. Correlation coefficients were also calculated to assess potential connections between measurements. The statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Protein hydrazones in MDA-treated protein suspensions

At a first stage, total protein carbonyls, as assessed by the DNPH method, were quantified in proteins incubated with MDA and their corresponding controls (no added MDA). The concentration of protein hydrazones (DNPH-derivatized carbonyls) in LAC, HSA and HEM incubated for 24 h at whether 80 °C (LAC) or 37 °C (human proteins) is displayed in Fig. 3. In control samples, the concentration of protein carbonyls increased from 0.6, 0.35 and 0.74 to 3.18, 0.81 and 1.25 nmol hydrazones/mg protein in LAC, HSA and HEM, respectively. Despite not adding a specific protein oxidation promoter, the carbonylation of proteins took place in control samples as a likely result of the formation of radical species from residual oxygen in the headspace of the vials (~20.5%). In accordance to calculations made in a similar previous study [17], a low concentration of $O_2^{\cdot-}$ in control samples (5 nM; thousand times lower than in Fenton-reaction systems) may be sufficient to initiate the oxidative deamination of alkaline amino acids and hence, to cause the formation of protein carbonyls. Consistently, other authors such as Akagawa et al. [15,21] and Gürbüz et al. [22] reported the carbonylation of HSA and other proteins while incubated at 37 °C in an oxygenated environment. The higher concentration of protein hydrazones in LAC compared to those in human proteins is explained by the severe temperature of incubation to which the former was subjected (compatible with milk-dairy product processing).

The incubation of proteins with MDA had a remarkable effect on the total amount of hydrazones in all the proteins under study. The notable concentrations of hydrazones in proteins incubated with MDA was already observed at the first sampling, right after MDA addition, and before subjecting the protein suspensions to the incubation temperatures (14.7, 3.2 and 8.7 nmol of hydrazones/mg protein in LAC, HSA and HEM, respectively). The subsequent incubation led to significant and remarkable increases of hydrazones in LAC and significant but more moderate increases of hydrazones in HSA and HEM. The increases in protein hydrazones occurred along with a depletion of free amino groups (Fig. 4), with this decrease being positively and highly correlated with the accretion of hydrazones ($r = 0.79$; $p < 0.05$). This timely coincidence provides strength to the plausible implication of MDA in both events. The addition of the carbonyl moieties in MDA to the nucleophilic ε-amino groups in alkaline amino acids in a Michael addition-type reaction could explain the decrease of the latter as compared to the control samples. In literature, this reaction is typically described to take place between MDA and its degradation products, acetaldehyde (AA) and formaldehyde (FA), with lysine to form adducts

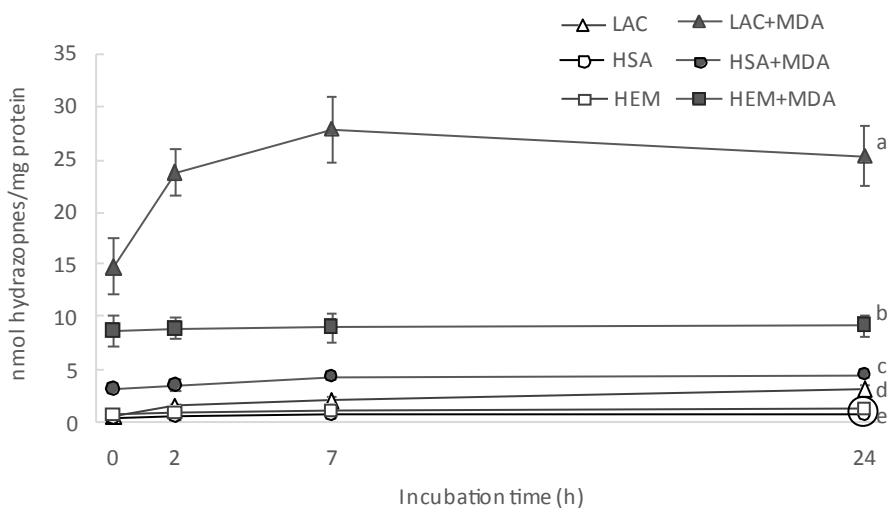

Fig. 3. Concentration of protein hydrazones (means ± standard deviations) during incubation of HSA, HEM and LAC for 24 h.
Footnote: For simplification purposes, only results from statistical analysis at final sampling point are displayed. Different letters denote significant differences ($p < 0.05$) among experimental units in a post-hoc Tuckey test. Samples grouped within a circle share the same letter.

of assorted stability [23]. The adduct formed between one lysine residue and one MDA molecule (Fig. 2A) is unstable, given the reactivity of the free aldehyde moiety. In fact, the addition of a second lysine residue yields and intra- and/or intermolecular covalent cross-linked 1-amino-3-iminopropene-type MDA-lysine adduct (2B), a final product of higher stability [23]. Other common reactions involve MDA, lysine and FA or AA (2:1:1), which also results in stable products such as 1,4-dihydropyridine-3,5-dicarbaldehyde ($[MDA]_2$-FA-Lys; Fig. 2C) and the reaction of four MDA with two lysines to yield 3,5-diformyl-1,4-dihydropyridine-4-yl-pyridinium (FHP; $[MDA]_4$-$[Lys]_2$), Fig. 2D). To gain further insight into the structures of these adducts, protein suspensions were analyzed for the emission of fluorescence by Schiff base structures by a spectroscopy procedure (Fig. 5). Evident similarities were found between the evolution of the formation of fluorescent Schiff base structures throughout the assay (Fig. 5) and the formation of protein hydrazones (Fig. 3). The positive and significant high correlation calculated between both measurements ($r = 0.91$; $p < 0.05$), provides strength to the hypothesis that MDA-lysine adducts not only had one carbonyl available for the addition of DNPH, but also they were able to emit natural fluorescence. These findings strongly incriminate hybrid and complex fluorescent MDA adducts such as $(MDA)_2$-FA-Lys (Fig. 2C) and $(MDA)_4$-$(Lys)_2$ (Fig. 2D) and rule out other linear and simpler adducts such as MDA-Lys (Fig. 2A) and MDA-$(Lys)_2$ (Fig. 2B) [24].

3.2. Formation of allysine in MDA-treated protein suspensions

Yet, one of our initial hypothesis was indefinite: is MDA, as a reactive dicarbonyl, able to induce oxidative deamination of lysine residues to yield allysine? This possibility was formulated as feasible given that other small molecular weight dicarbonyls such as GO and MGO have been proven to trigger this Maillard-type mechanism [7]. As displayed in Fig. 1, firstly, the dicarbonyl is added to the ε-amino group in a protein-bound lysine to yield an unstable Schiff base which is comparable to the MDA-Lys adduct depicted in Fig. 2A. Subsequently, the enolization of the second carbonyl moiety leads to the formation of an iminoenaminol which is spontaneously hydrolyzed to release an enaminol and yield allysine. To our knowledge, these last stages have never been described to occur upon the reaction between MDA and lysine residues. However, this reaction would explain all the results reported so far: the depletion of free amino groups in proteins and the subsequent formation of hydrazones through the derivatization of allysine by the DNPH. To elucidate whether our hypothesis was correct or not, protein suspensions incubated with MDA were analyzed for the formation of allysine. Allysine was found to increase in control samples, which is consistent with the DNPH results (Fig. 6). In fact, allysine is the most abundant carbonyl in proteins and accounts between 40 and 70% of total protein hydrazones in HSA, BSA and assorted food proteins [7,15,20,25]. Contradicting our initial hypothesis, allysine concentrations remained unchanged during the incubation of protein suspensions

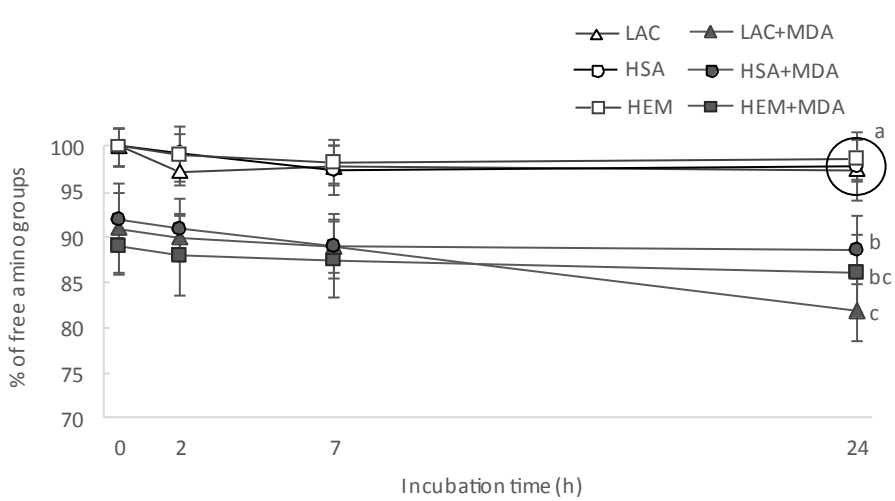

Fig. 4. Concentration of free amines (means ± standard deviations) during incubation of HSA, HEM and LAC for 24 h.
Footnote: For simplification purposes, only results from statistical analysis at final sampling point are displayed. Different letters denote significant differences ($p < 0.05$) among experimental units in a post-hoc Tuckey test. Samples grouped within a circle share the same letter.

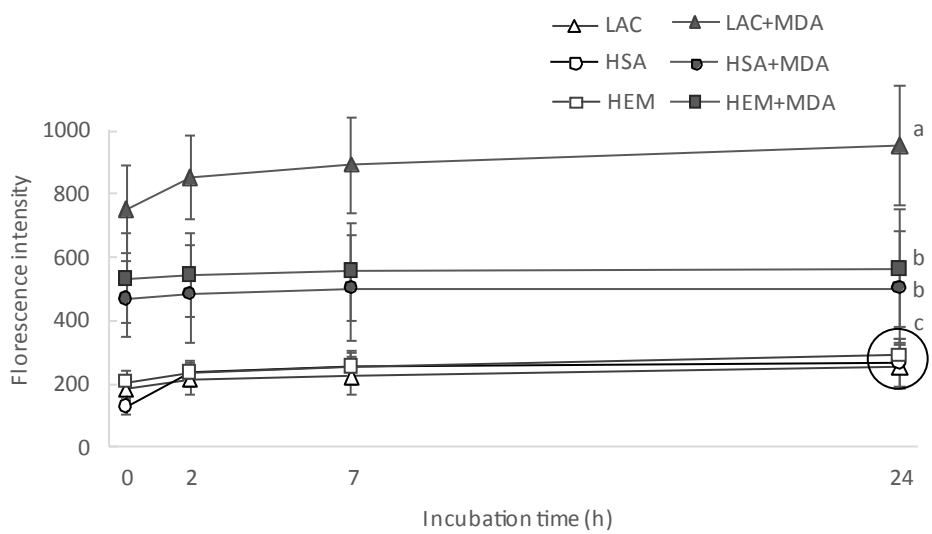

Fig. 5. Fluorescence intensity emitted by Schiff bases structures (means ± standard deviations) during incubation of HSA, HEM and LAC for 24 h.
Footnote: For simplification purposes, only results from statistical analysis at final sampling point are displayed. Different letters denote significant differences ($p < 0.05$) among experimental units in a post-hoc Tuckey test. Samples grouped within a circle share the same letter.

with MDA (Fig. 6). Therefore, under the conditions of the present experiment, MDA is unable to induce the oxidative deamination of lysine residues. Hence, the second aldehyde moiety from MDA would not undergo enolization and instead may fulfil its electrophilic nature by adding to additional amino groups as reported above. Furthermore, the addition of MDA to the ε-amino groups impedes the subsequent oxidative deamination of lysine by any means (radical-mediated or α-dicarbonyl-mediated): the formation of allysine is hindered when MDA is present.

The findings from the present study confirm that MDA would account for protein hydrazones when the DNPH is used as a routine method for the detection of protein carbonyls. This has relevant consequences from a scientific point of view because the DNPH method is typically used to assess protein carbonylation as the most salient expression of protein oxidation in biological samples. Ever since the DNPH method was applied to quantify protein carbonyls, these compounds have been praised to be the result of a metal-catalyzed and radical-mediated oxidative damage to proteins and hence, indicators of an on-site protein carbonylation [26,27]. In their highly influential paper, Levine et al. [27] performed a detailed description of the method and emphasized its significance to identify oxidatively modified proteins and highlighted γ-glutamyl semialdehyde (GGS, oxidation product from arginine and proline) as the most abundant protein carbonyl. In subsequent revisions of the method, the same authors [13,28] distinguished between primary (~oxidation of amino acid residues into carbonyls) and secondary protein carbonylation (~introduction of preformed carbonyls into proteins). Nevertheless, in further review papers, GGS and allysine are stated as the main carbonyl products in proteins and the DNPH method emphasized as an accurate technique for their detection [6]. Requena et al. [6] regarded secondary carbonylation as a negligible event limited to rat liver samples in which carbonyls may also be introduced by glycation and lipid peroxidation products. In their recent review, Alomari et al. [29] refer to the 'Levine' DNPH method as a valid biomarker of oxidatively stressed proteins. Relevant papers in the field are discriminated between those in which the complex nature of protein carbonyls is emphasized and the validity of the DNPH method as a precise technique for assessing oxidized proteins is questioned [25,30–32] and those in which such complexity is overlooked and protein hydrazones are assumed to mainly reflect primary oxidation of amino acid residues in proteins [33,34]. The present results confirm that such an assumption leads to misleading conclusions. Whenever lipid oxidation is concurrent to the oxidative damage to proteins, MDA and likely, other lipid-derived carbonyls, are introduced in proteins by reacting with ε-amino groups. Furthermore, depending on the oxidation conditions and mechanisms, if lipid oxidation is intense and MDA is profusely formed, the nucleophilic addition of MDA

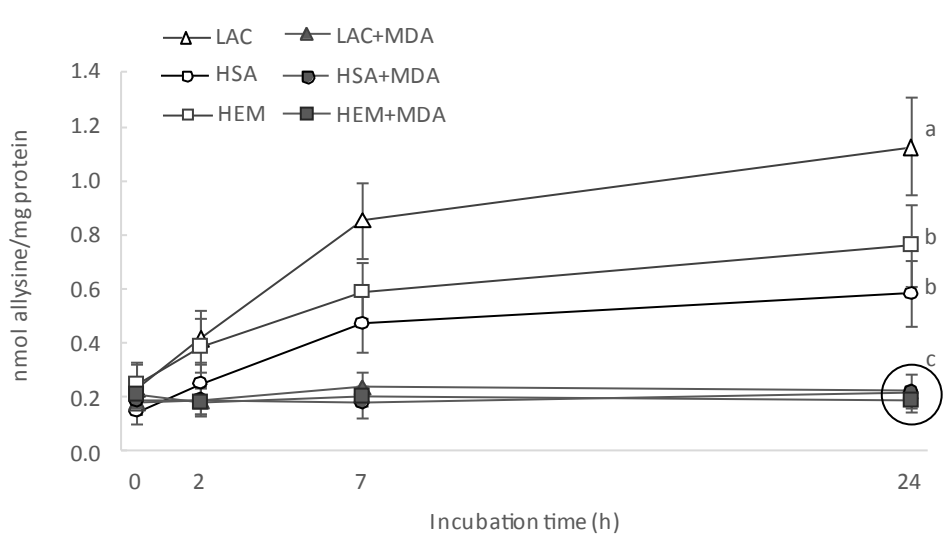

Fig. 6. Concentration of allysine (means ± standard deviations) during incubation of HSA, HEM and LAC for 24 h.
Footnote: For simplification purposes, only results from statistical analysis at final sampling point are displayed. Different letters denote significant differences among experimental units in a post-hoc Tukey test. Samples grouped within a circle share the same letter.

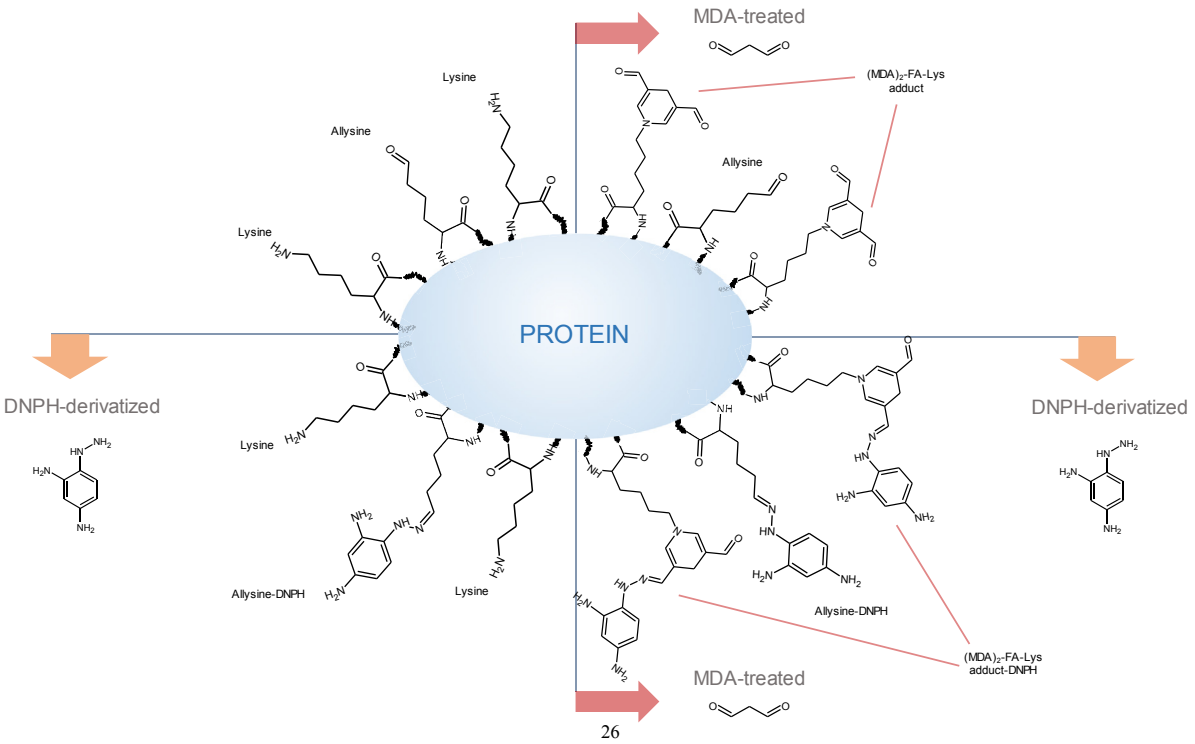

Fig. 7. Illustration of protein bound lysines and allysines in the presence and/or absence of MDA and DNPH.

to proteins will hamper the primary oxidation of alkaline amino acids such as lysine, and the subsequent formation of allysine. Fig. 7 aims to depict how MDA impedes the formation of allysine and yet, contribute to the formation of protein hydrazones upon reaction with the DNPH reagent. Nevertheless, the DNPH method specifically reflects the carbonylation of proteins but the biochemical mechanism behind that oxidative modification is so complex and variable that accurate and in-depth scientific discussion are not allowed unless other and more specific methodologies are applied to biological samples.

3.3. Dose-effect of MDA on HSA carbonylation

A subsequent assay, aimed to assess the effect of MDA concentration on the events under examination, showed a dose-dependent interfering effect of MDA on allysine formation in the range between 0.05 and 1 mM. Increasing MDA concentration led to accumulative accretion of protein hydrazones (Fig. 8A), subsequent depletion of free amino groups (Fig. 8B) and increasing gain of Schiff base fluorescence (Fig. 8C). Conversely, the formation of allysine declined as the MDA concentration increased, providing strength to the hypothesis of MDA forming adducts with ε-amino groups in protein-bound lysines, and as a result, hindering the oxidative deamination of this amino acid. It is worth highlighting that this interfering effect was observed at biologically relevant MDA concentrations (0.05 mM) [11,12] and the dose-effect was detected at concentrations that may also be relevant in food systems (1 mM). The formation of allysine, already at basal concentrations at 1 mM, was not diminished at higher MDA concentrations. Yet, a complete saturation of amino groups was only observed at 5 mM which is reasonable considering that other amino acids (different from lysine) may also be present and do not yield allysine. Above 5 mM of MDA, no significant changes were observed, with those concentrations being out of the expected range of MDA concentration in biological samples (including food systems) [4].

4. Conclusions

The DNPH is a valid method to assess protein carbonylation caused through a variety of pathways and mechanisms, including: i) ROS-mediated oxidation; ii) Maillard-mediated glycooxidation by reducing sugars and their oxidation products and iii) the addition of pre-formed lipid oxidation-aldehydes. While all of them lead to the occurrence of protein carbonyls, the two first mechanisms involve the oxidation of alkaline amino acids and the formation of primary protein carbonyls (such as allysine), and the last mechanism involves the Michael-type addition of pre-formed carbonyls such as MDA. The present study proves that under severe MDA-mediated stress, the formation of primary protein carbonyls is blocked and most protein carbonyls assessed by the DNPH method would reflect the oxidative damage caused by lipid oxidation products on proteins. Being aware of its limitations, protein scientists should accurately interpret results from this commonly used method, and apply, when required, other methodologies such as chromatographic methods to detect specific primary oxidation products such as allysine.

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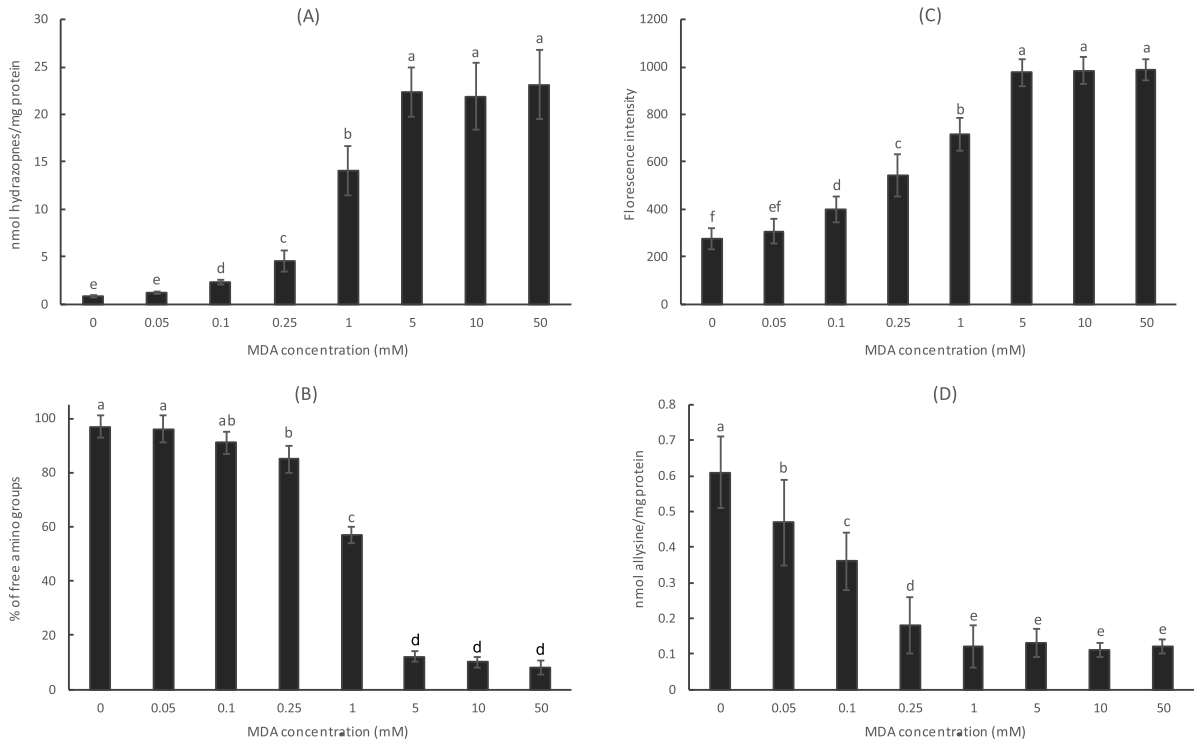

Fig. 8. Effect of MDA concentration on accretion of protein hydrazones (A), loss of free amino groups (B), fluorescence gain (C) and concentration of allysine (D) (means ± standard deviations) after incubation with HSA for 24 h.
Footnote: Different letters on top of bars denote significant differences among MDA concentration in a post-hoc Tukey test.

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4. III: An *in vitro* assay of the effect of lysine oxidation end-product, α-aminoadipic acid, on the redox status and gene expression in probiotic *Lactobacillus reuteri* PL503.

Un ensayo in vitro del efecto del producto final de la oxidación de la lisina, el ácido α-aminoadípico, sobre el estado redox y la expresión génica en el probiótico *Lactobacillus reuteri* PL503

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ORIGINAL ARTICLE

An in vitro assay of the effect of lysine oxidation end-product, α-aminoadipic acid, on the redox status and gene expression in probiotic *Lactobacillus reuteri* PL503

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Abstract
This study was designed to gain information about the underlying mechanisms of the effects of a food-occurring free oxidized amino acid, α-aminoadipic acid (AAA), on the probiotic *Lactobacillus reuteri* PL503. This bacterium was incubated in colonic-simulated conditions (37 °C for 24 h in microaerophilic conditions) and exposed to three food-compatible AAA concentrations, namely, 1 mM, 5 mM, and 10 mM. A control group with no AAA exposure was also considered. Each of the four experimental conditions was replicated three times and samplings were collected at 12, 16, 20, and 24 h. The down-regulation of the *uspA* gene by AAA (0.5-fold decrease as compared to control) suggests that AAA is identified as a potential chemical threat. The *dhaT* gene, implicated in the antioxidant defense, was found to be upregulated in bacteria treated with 1 and 5 mM AAA (up to twofold increase, as compared to control), which suggest the ability of the oxidized amino acid to impair the redox status of the bacterium. In fact, AAA caused an increased production of reactive oxygen species (ROS) and the accretion of post-translational changes (protein carbonylation) in *L. reuteri* (up to 13 nmol allysine/mg protein vs 1.8 nmol allysine/mg protein in control). These results suggest that probiotic bacteria identify oxidized amino acids as harmful species and activate mechanisms that may protect themselves and the host against their noxious effects.

Keywords Oxidized amino acids · Oxidative stress · Probiotic bacterium · Protein oxidation · Transcripts

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Abbreviations

1,3-PDO	Propane-1,3-diol
3-HPA	3-Hydroxypropionaldehyde
AAA	α-Aminoadipic acid
AAS	α-Aminoadipic semialdehyde
ANOVA	Analyses of variance
DNA	Deoxyribonucleic acid
ABA	Aminobenzoic acid
DPS	4,4′-Dipyridyldisulphide
DTPA	Diethylenetriaminepentaacetic acid
FLD	Fluorescence detector
FMO	Fluorescence minus one
HPLC	High-performance liquid chromatography
MDA	Malondialdehyde
MES	2-(N-morpholino)ethanesulfonic acid
MRS	Man Rogosa and Sharpe
NADH	Nicotinamide adenine dinucleotide
PBS	Phosphate buffered saline solution
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
RNA	Ribonucleic acid
TBARS	Thiobarbituric-reactive substances

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TCA	Trichloroacetic acid
TEP	Tetraethoxypropane
UspA	Universal stress protein A

Introduction

Protein oxidation is a post-translational modification induced by reactive oxygen species (ROS) and other pro-oxidative compounds, and plays an essential role in the pathogenesis of relevant degenerative diseases (Davies 2005). The oxidative damage to proteins leads to depletion of original amino acids and the formation, in its place of specific oxidation products (Davies 2005). Chemical species such as the α-aminoadipic semialdehyde (AAS), also known as allysine, and its end-product, the α-aminoadipic acid (AAA), are generated by the oxidation of lysine through metal-catalyzed reactions (Stadtman and Oliver 1991). While both species occur as intermediates in lysine metabolism, the accretion of such species in biological samples, including food systems, respond to a radical-mediated oxidation mechanism (Davies 2005; Stadtman and Oliver 1991). The chemical structures and formation mechanisms of these oxidized amino acids can be found in detail elsewhere (Luna et al. 2021). AAA has been identified in meat products, such as raw and cooked patties, cooked sausages and fermented meats, at levels ranging 50–200 μM (Utrera and Estévez 2012; Utrera et al. 2012). Recently, Estévez and Xiong (2019) collected information about the scientific evidences of the potential harmful effects of dietary oxidized proteins and amino acids. AAA, in particular, has been found to exert, at food-compatible concentrations of AAA (200 μM), mitochondrial disturbance, oxidative stress, apoptosis, and necrosis in human intestinal and mice pancreatic cells (Díaz-Velasco et al. 2020; Estaras et al. 2020).

Among the assorted pathophysiological conditions induced by the intake of oxidized proteins and amino acids, the disturbance of the microbiota has been found in both in vitro (Arcanjo et al. 2019) and in vivo (Goethals et al. 2020) studies. The protective role of microbiota is gaining interest since luminal oxidative stress in humans can be counteracted by microbiota (Spyropoulos et al. 2011). In this regard, *Lactobacillus reuteri*, a natural colonizer of the gastrointestinal tract in humans and animals, has been used as a dietary supplement to enhance human gut health (Shornikova et al. 1997), and its oral administration reduces gastrointestinal disorders and infections and contributes to a balanced colonic microbiota (Shornikova et al. 1997). *L. reuteri* has been reported to protect against oxidative stress and inhibits the accretion of oxidation products in the lumen, according to the mechanisms related to its probiotic effects (Amaretti et al. 2013). While the benefits of *L. reuteri* against oxidative stress are documented (Petrella 2016), the molecular mechanisms implicated in the responses of this probiotic bacterium under specific pro-oxidant conditions are not well understood.

According to some previous reports, the expression of the *uspA* and *dhaT* genes in *L. reuteri* is affected by the oxidative threat caused by reactive oxygen species (ROS) (Arcanjo et al. 2019). Usp proteins seem to be implicated in the defense against DNA-damaging agents while the *dhaT* gene encodes for a propane-1,3-diol (1,3-PDO) oxidoreductase, which is involved in the protection of *L. reuteri* against oxidative stress (Arcanjo et al. 2019). In a preceding study, we investigated the molecular responses of this bacterium to a major lipid oxidation product, malondialdehyde (MDA), at concentrations between 5 and 100 μM (Padilla et al. 2021). Yet, the underlying mechanisms of the potential impact of oxidized amino acids on probiotic bacteria are unknown.

The aim of this study was to understand the molecular mechanisms activated in *L. reuteri* in response to the potential harmful effects of AAA. To fulfil this objective, the redox status (ROS generation and lipid and protein oxidation markers), and the expression of the *uspA* and *dhaT* genes in *L. reuteri* challenged by increasing concentrations of AAA, was investigated.

Materials and methods

Chemicals and raw material

Chemicals and reagents used in this study were of American Chemical Society analytical grade and purchased from Sigma Chemicals (Sigma–Aldrich, Germany), Scharlab S.L. (Spain), Pronadisa (Conda Laboratory, Spain), Applied Biosystems (USA), Epicentre (USA), and Acros Organics (Spain). *L. reuteri* PL503 was isolated from pig faeces and then identified using 16S rRNA gene sequencing by Ruiz-Moyano et al. (2008).

Experimental setting

L. reuteri PL503 was stored at − 80 °C in Man Rogosa and Sharpe (MRS) broth with 20% (v/v) glycerol. To prepare the working cultures, *L. reuteri* PL503 was cultivated twice at 37 °C for 24 h in MRS broth supplemented with 0.5% acetic acid 10% (v/v). A volume of 100 μL of such culture of *L. reuteri* PL503 was inoculated in tubes of 5 mL of MRS broth containing different concentrations of free AAA. In particular, four groups were considered based on the added concentration of free AAA: Control (*L. reuteri*), 1 mM (*L. reuteri* + 1 mM AAA), 5 mM (*L. reuteri* + 5 mM AAA), and 10 mM (*L. reuteri* + 10 mM AAA). They were incubated at 37 °C for up to 24 h in microaerophilic conditions to simulate physiological conditions in the colon. The

concentrations of free AAA are those expected to be found in the colon after gastrointestinal digestion of a severely processed muscle food (Utrera and Estévez 2012; Utrera et al. 2012; Goethals et al. 2020). For each treatment, three replicates were performed. During the incubation period, samples were taken at 12, 16, 20, and 24 h. For counting viable cells, 100 µL of *L. reuteri* PL503 of each treatment and sampling time were inoculated on MRS agar at the same sampling time and conditions as the experimental tubes. For protein analyses, to avoid possible contamination from the culture medium, two washes with phosphate buffered saline solution (PBS, pH 7.4) were made.

Gene expression studies

RNA extraction

The RNA extraction of each experimental group and incubation time was performed using the MasterPure™ RNA purification kit (Epicentre), which includes DNase treatment. The obtained RNA was eluted in 35 µL TE buffer and kept at − 80 °C until further use. RNA quantity (ng/µL) and quality (A_{260}/A_{280} ratio) were spectrophotometrically determined using the Nanodrop 2000 (Thermo Scientific, USA).

Reverse transcription reaction

The cDNA was synthesized using about 500 ng of total RNA, according to the PrimeScript™ RT Reagent kit (Takara Bio Inc., Japan). The cDNA was stored at − 20 °C until being used for the PCR reactions.

Real-time PCR analysis of gene expression

The *uspA* and *dhaT* genes were selected for relative expression studies using real-time PCR (qPCR), being used the 16S gene as reference gene. The amplification was performed in MicroAmp optical 96-well plates sealed with optical adhesive covers (Applied Biosystems) on a ViiA™ 7 Real-Time System (Applied Biosystems) using the SYBR Green technology. Each well contained 2.5 µL of cDNA, 6.25 µL of SYBR® Premix Ex Taq™ (Takara Bio Inc.), 0.625 µL of ROX™ Reference Dye (Takara Bio Inc.), and 300 nM of each primer pair (Table 1). The qPCR program consisted of an initial denaturation step at 95 °C for 10 min; 40 cycles at a denaturation temperature of 95 °C for 15 s and annealing/extension temperatures of 55 °C and 60 °C for the 16S and target genes, respectively, during 30 s. After the final qPCR cycle, a melting curve was included by heating the product from 60 to 99 °C and continuous measurement of the fluorescence was performed to verify the qPCR products. All samples were analyzed in triplicate, including control samples consisting of adding sterile ultrapure water instead of cDNA. The expression ratio was calculated using the $2^{-\Delta\Delta C_T}$ method reported by Livak and Schmittgen (2001). The calibrator sample corresponded to the value of the expression of the experimental group Control at each sampling time.

Analytical procedures

Analysis of ROS by flow-cytometry

Flow cytometry detection of ROS (e.g., hydroxyl and superoxide radicals) in *L. reuteri* PL503 was performed as determined using protocols described by Díaz-Velasco et al. (2020) with some minor modifications. In brief, samples of *L. reuteri* PL503 (1×10^6 ufc/mL) of each experimental group and incubation time, were extended in 1 mL of PBS, and stained with CellRox Deep Red (5 µM; ThermoFisher, USA) (excitation and emission wavelengths, 644 and 645 nm, respectively) for detecting the bacterium producing ROS, and Hoechst 33,342 (0.5 µM; Sigma–Aldrich) (excitation and emission wavelengths, 345 nm and 488 nm, respectively) to identify the bacterium and remove debris from the analysis. After thorough mixing, the cell suspension was incubated at room temperature for 25 min in the dark, washed in PBS and immediately run on the flow cytometer. The analyses were conducted using a Cytoflex® flow cytometer (Beckman Coulter, USA) equipped with violet, blue, and red lasers. The instrument was daily calibrated using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each

Table 1 Sequences of primers used for reverse transcription real-time PCR assays to conduct gene expression analyses

Primers	Gene	Nucleotide sequence (5′-3′)	Annealing temperature	References
uspALr-F1	*uspA*	CTTGGGTAGCGTTCACCATT	60 °C	Arcanjo et al. (2019)
uspALr-R1		TGAAAAAGCGGTTGACACTG	60 °C	Arcanjo et al. (2019)
LS67	*dhaT*	TGACTGGATCCTAATTTGGTCCTGGTGTTATTGC	60 °C	Schaefer et al. (2010)
LS68		TGACTGAATTCTTCCGGATCTTAGGGTTAGG	60 °C	Schaefer et al. (2010)
Lr16S_F	16S rRNA	CCGCTTAAACTCTGTTGTTG	55 °C	Arcanjo et al. (2019)
Lr16S_R		CGTGACTTTCTGGTTGGATA	55 °C	Arcanjo et al. (2019)

experiment; however, due to emission and excitation characteristics of the combination of the used probes, spectral overlap was negligible. Files were exported as FCS files and analyzed using FlowJoV 10.5.3 Software for Mac OS (Ashland, USA). Unstained, single-stained, and Fluorescence Minus One (FMO) controls were used to determine compensations and positive and negative events, as well as to set regions of interest.

Synthesis of allysine standard compound

N-Acetyl-L-AAS (allysine) was synthesized from Nα-acetyl-L-lysine using lysyl oxidase activity from egg shell membrane following the procedure described by Akagawa et al. (2002). Briefly, 10 mM Nα-acetyl-L-lysine was incubated at constant stirring with 5 g of egg shell membrane in 50 mL of 20 mM sodium phosphate buffer, pH 9.0 at 37 °C for 24 h. The egg shell membrane was then removed by centrifugation and the pH of the solution adjusted to 6.0 using 1 M HCl. The resulting aldehydes were reductively aminated with 3 mM 4-aminobenzoic acid (ABA) in the presence of 4.5 mM sodium cyanoborohydride ($NaBH_3CN$) at 37 °C for 2 h with stirring. ABA derivatives were then hydrolyzed by 50 mL of 12 M HCl at 110 °C for 10 h. The hydrolysates were evaporated at 40 °C *in vacuo* to dryness. The resulting allysine–ABA was purified using silica gel column chromatography and ethyl acetate/acetic acid/water (20:2:1, v/v/v) as elution solvent. The purity of the resulting solution (70%) and authenticity of the standard compounds obtained following the aforementioned procedures were checked using MS and ^{1}H NMR (Estévez et al. 2009).

Quantification of allysine

Allysine was quantified in bacterial protein as a marker of oxidation-induced post-translational modification, according to the method described by Utrera et al. (2011). Five hundred μL of each experimental group and incubation time of culture were dispensed in 2 mL microtubes and treated with cold 10% (v/v) trichloroacetic acid (TCA) solution. Each microtube was vortexed and then subjected to centrifugation at 600 × *g* for 5 min at 4 °C. The supernatants were removed, and the pellets were incubated with the following freshly prepared solutions: 0.5 mL 250 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 6.0 containing 1 mM diethylenetriaminepentaacetic acid (DTPA), 0.5 mL 50 mM ABA in 250 mM MES buffer pH 6.0 and 0.25 mL 100 mM $NaBH_3CN$ in 250 mM MES buffer pH 6.0. After vortexing, the tubes were incubated in a water bath at 37 °C for 90 min. The samples were stirred every 15 min. The samples were then treated with a cold 50% TCA (v/v) solution and centrifuged at 1200 × *g* for 10 min. The pellets were washed twice with 10% TCA and diethyl ether-ethanol (1:1). Finally, the pellet was treated with 6 M HCl and kept in an oven at 110 °C for 18 h until completion of hydrolysis. The hydrolysates were dried *in vacuo* in a centrifugal evaporator. The generated residue was reconstituted with 200 μL of milliQ water and then filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 μm pore size, Pall Corporation, USA) for HPLC analysis.

A Shimadzu 'Prominence' HPLC apparatus (Shimadzu Corporation, Japan), equipped with a quaternary solvent delivery system (LC-20AD), a DGU-20AS on-line degasser, a SIL-20A auto-sampler, a RF-10A XL fluorescence detector (FLD), and a CBM-20A system controller, was used. An aliquot (1 μL) from the reconstituted protein hydrolysates was injected and analyzed in the HPLC-FLD equipment. Allysine–ABA was eluted in a Cosmosil $5C_{18}$-AR-II RP-HPLC column (5 μm, 150 × 4.6 mm) equipped with a guard column (10 × 4.6 mm) packed with the same material (Phenomenex, PA, USA). The flow rate was kept at 1 mL/min and the temperature of the column was maintained constant at 30 °C. The eluate was monitored with excitation and emission wavelengths set at 283 and 350 nm, respectively. Standards (0.1 μL) were run and analyzed under the same conditions. Identification of both derivatized semialdehydes in the chromatograms was carried out by comparing their retention times with those from the standard compounds. The peak corresponding to allysine–ABA was manually integrated from the FLD chromatograms and the resulting areas plotted against an ABA standard curve with known concentrations that ranged from 0.1 to 0.5 mM (Utrera et al. 2011). Results were expressed as nmol of allysine per mg of protein.

Analysis of Schiff bases

The formation of Schiff bases in bacterial protein was assessed in each experimental group and incubation time by fluorescence spectroscopy. Prior to the analysis, reaction mixtures were diluted (1:20) with 8 M urea in 100 mM sodium phosphate buffer, pH 7. Diluted samples were dispensed in spectrofluorometric cuvettes and excited at 350 nm using a LS-55 Perkin–Elmer fluorescence spectrometer (PerkinElmer, UK). The fluorescence emitted by Schiff bases was recorded at 450 nm. The excitation and emission slit widths were set at 10 nm and the speed of data collection while scanning was of 500 nm per min. The height of the peaks corresponding to Schiff bases spectra was recorded. After taking into consideration the applied dilutions, the results were expressed as fluorescence units.

Analysis of protein thiols

Thiols from sulfur-containing amino acids in bacterial proteins were quantified in accordance to the method reported

by Rysman et al. (2014). A volume of 250 µL of each *L. reuteri* PL503 experimental group and incubation time, was washed twice with PBS and ethanol:ethyl acetate (1:1) to avoid possible contamination with thiols from the medium. Upon centrifugation (600 × *g*/5 min), the pellet was resuspended in 250 µL of guanidine hydrochloride, treated with 250 µL of 4,4′-dipyridyldisulphide (DPS) in 12 mM HCl and dispensed in a spectrophotometric cuvette. Absorbance was measured at 324 nm against a blank sample in which DPS was replaced with an equivalent volume of guanidine hydrochloride. Quantification was made by preparing a standard curve with cysteine. The results were expressed as µmol of free thiol groups per mg of protein.

Analysis of thiobarbituric-reactive substances

The quantification of MDA and other thiobarbituric-reactive substances (TBARS) in all experimental groups and incubation times, was made in accordance to the method described by Ganhao et al. (2011). An aliquot of 200 µL of *L. reuteri* PL503 experimental group was treated with 500 µL of thiobarbituric acid (0.02 M) and 500 µL of TCA (10%) and incubated during 20 min at 90 °C. After cooling, a 5 min centrifugation at 600 × *g* was made and the supernatant was measured at 532 nm. Quantification was made by preparing a standard curve with tetraethoxypropane (TEP). The results are expressed as mg TBARS per L of sample.

Statistical analysis

True replicates ($n = 3$) were subjected to duplicate analyses and data were collected and subjected to statistical analysis. Earlier, the data were analyzed for normality (Shapiro–Wilk test) and homoscedasticity (Bartlett test). The effects of AAA concentration and incubation times were studied using analyses of variance (ANOVA) (SPSS v. 15.5). The effect of AAA on the gene expression ($\Delta\Delta C_T$ values) was analyzed using the paired Students' *t* test (SPSS v. 15.5). The statistical significance was set at $p \leq 0.05$.

Results

Relative expression of the uspA gene

The relative expression of the *L. reuteri* PL503 *uspA* gene during the incubation assay in the presence of different concentrations of AAA is shown in Fig. 1a. A significant downregulation of the *uspA* gene was particularly observed at 12 h of incubation in the presence of 5 and 10 mM of AAA (0.22- and 0.40-fold decrease, respectively) as well

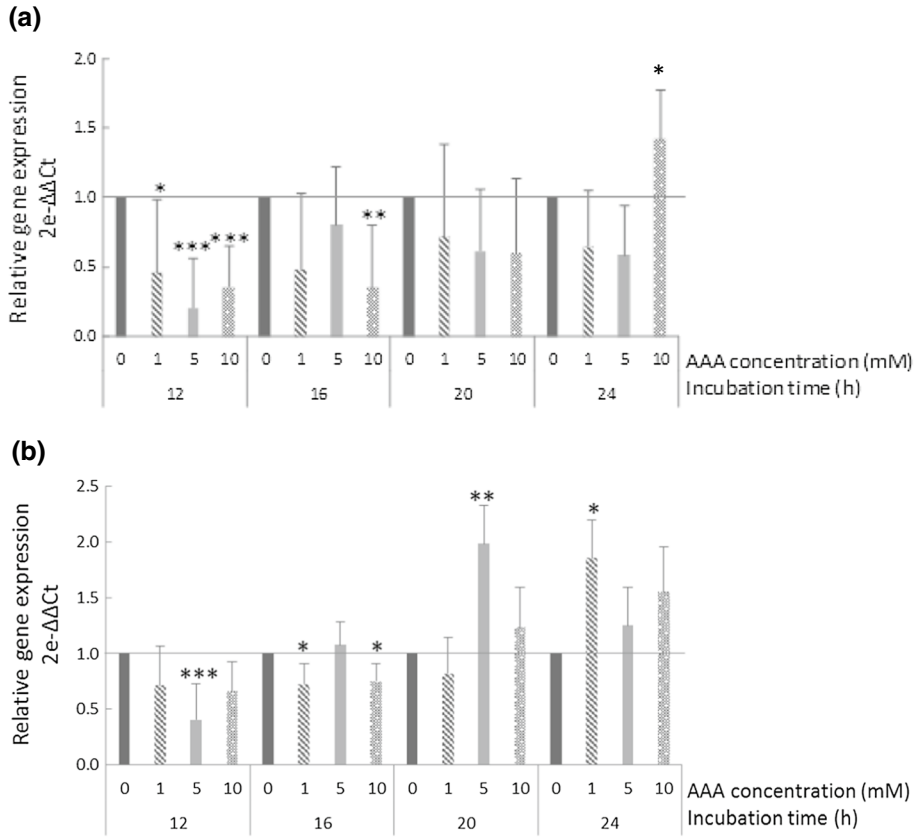

Fig. 1 Relative expression ($2^{-\Delta\Delta C_T}$) of the *uspA* (**a**) and *dhaT* (**b**) genes in *Lactobacillus reuteri* PL503 grown in the presence of increasing concentrations (0, 1, 5, and 10 mM) of α-aminoadipic acid (AAA) for up to 24 h. Black line at $2^{-\Delta\Delta C_T} = 1$ denotes standardized expression rate for CONTROL group (0 mM) at each sampling time (calibrator). $2^{-\Delta\Delta C_T} < 1$ denotes suppression of the expression of the target gene; $2^{-\Delta\Delta C_T} > 1$ denotes activation of the expression of the target gene. Asterisks on top of bars denote significant differences between such treatment and the control within a particular sampling time (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$)

as at 16 h sampling with 10 mM (0.38-fold decrease). An upregulation of the gene (1.43-fold increase) was observed at 24 h when the bacterium was exposed to the highest AAA concentration.

Relative expression of the *dhaT* gene

The relative expression of the *L. reuteri* PL503 *dhaT* gene during the incubation assay in the presence of different concentrations of AAA is shown in Fig. 1b. A significant downregulation of the gene was found at 12 h in the presence of 5 mM (0.42-fold decrease) and at 16 h in the presence of both 1 mM and 10 mM of AAA (0.75- and 0.81-fold decreases, respectively). Nonetheless, in the two final sampling times, an upregulation of the relative transcription of the *dhaT* gene was observed. In particular, significant changes were found in the presence of 5 mM of AAA at 20 h (1.98-fold increase) and 1 mM of AAA at 24 h (1.83-fold increase).

ROS generation by flow-cytometry analyses

The incubation of *L. reuteri* PL503 in the presence of AAA led to an increased production of ROS as shown in Fig. 2. The analysis of the samples with flow-cytometry showed a clear dose effect. At increasing concentrations of AAA, the percentage of bacterium suffering oxidative stress at 24 h rise from 0.8% in control group to 1.8%, 2.1%, and 5.3% in bacteria exposed to 1, 5 and 10 mM AAA, respectively. Specially, at the two final sampling times, the differences between groups were found to be higher than in the previous ones.

Analysis of thiobarbituric-reactive substances

In Fig. 3a, the TBARS concentration in *L. reuteri* PL503 during the assay is shown. In the presence of AAA, significant changes occur at 20 h with 10 mM of AAA lowering TBARS content compared to control samples (0.89 mg TBARS/L vs. 1.10 mg TBARS/L). At 24 h, the concentration of TBARS in control samples (1.17 mg TBARS/L) was significantly higher than in the bacterium challenged with AAA (ranging from 1.05 to 1.10 mg TBARS/L).

Quantification of allysine

The changes of the concentration of allysine in *L. reuteri* PL503 during the incubation period is shown in Fig. 3b. Compared to control, the exposure to AAA caused a significant increase in the concentration of allysine in proteins from *L. reuteri* PL503 for 20 h. At that sampling point, the concentration of allysine in control samples (1.8 nmol/mg protein) was significantly lower than in those treated with 1, 5, and 10 mM AAA (11.7, 10.4, and 8.8 nmol/mg protein, respectively). At 24 h sampling, the behavior varied between groups. In *L. reuteri* challenged with 1 and 5 mM of AAA, the increase of allysine was constant during the complete assay reaching the highest concentration at 24 h (12.0 and 13.5 nmol/mg protein, respectively). On the other hand, when the bacterium was exposed to the highest AAA concentration (10 mM) allysine peaked at 20 h, after which a decrease was observed at the end of the incubation period (4.2 nmol/mg protein).

Analysis of Schiff bases

In the present study, the formation of Schiff bases is shown in Fig. 3c and a clear dose effect of AAA was observed. No significant differences were found between AAA concentrations during the first three sampling times. Nevertheless, at the final sampling time (24 h) an increase was observed when the bacterium was exposed to the highest concentration (10 mM), which is coincident with carbonyls depletion found in the same group of samples at the end of the assay. At 24 h, the relative concentration of Schiff bases in *L. reuteri* followed the increasing order: control group (52 fluorescent units) and bacterium exposed to 1, 5 and 10 mM AAA (80, 98, and 185 fluorescent units).

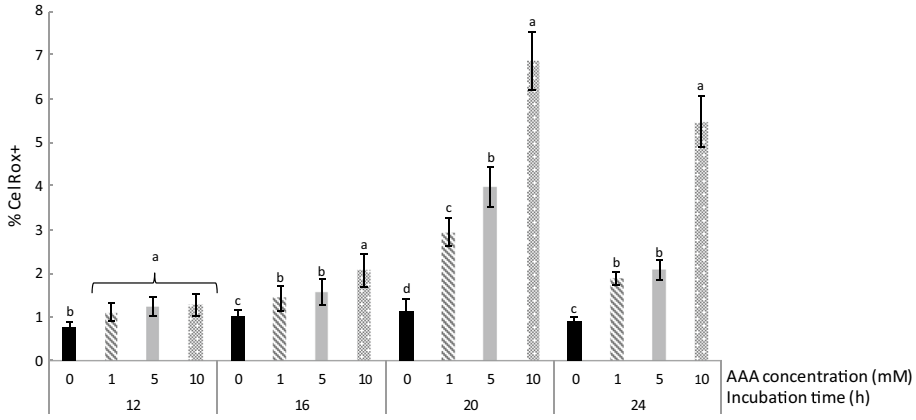

Fig. 2 Percentage of *Lactobacillus reuteri* PL503 suffering from oxidative stress (positive to Cell Rox dye) when grown in the presence of increasing concentrations (0, 1, 5 and 10 mM) of α-aminoadipic acid (AAA) for up to 24 h. Different letters on top of bars denote significant differences ($p \leq 0.05$) between AAA concentrations within the same sampling time

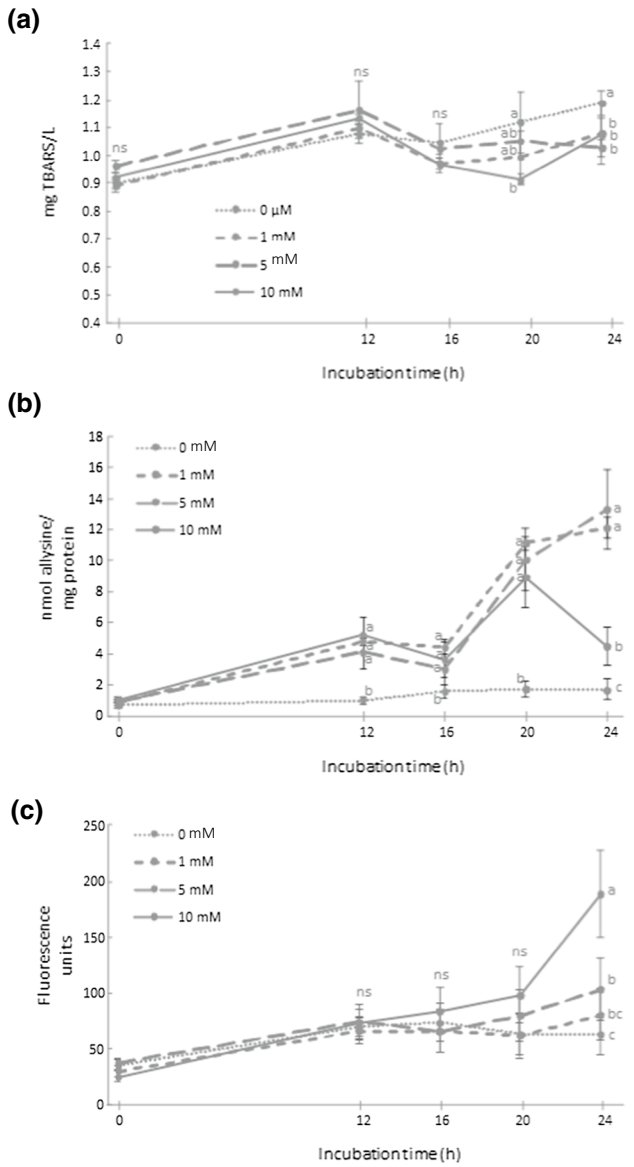

Fig. 3 Concentration of thiobarbituric-reactive substances (TBARS) (**a**), allysine (**b**) and Schiff bases (**c**) (means ± standard deviation) in *Lactobacillus reuteri* PL503 grown in MRS broth in the presence of increasing concentrations (0, 1, 5 and 10 mM) of α-aminoadipic acid (AAA) during an incubation period for up to 24 h. Different letters at the same sampling time denote significant differences between AAA concentrations ($p \leq 0.05$)

Analysis of protein thiols

The concentration of free thiols in proteins from *L. reuteri* PL503 during the incubation assay is shown in Fig. 4. Significant differences were observed in the first 12 h between the control group and the bacterium challenged with increasing AAA concentrations. From 16 h sampling time onwards, a significant increase of free thiols in samples exposed to 1 and 5 mM of AAA was detected, peaking at

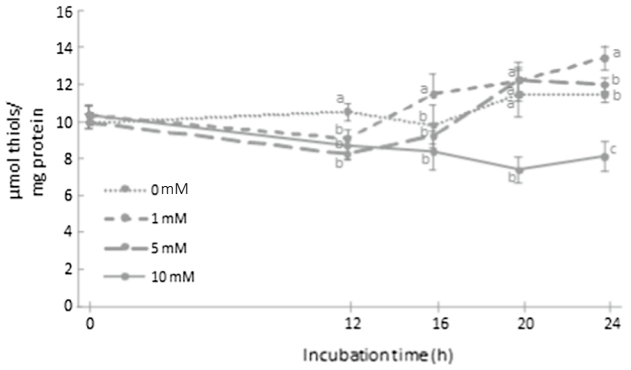

Fig. 4 Concentration of free thiols (means ± standard deviation) in *Lactobacillus reuteri* PL503 grown in MRS broth with increasing concentrations (0, 1, 5, and 10 mM) of α-aminoadipic acid (AAA) during an incubation period for up to 24 h. Different letters at the same sampling time denote significant differences between AAA concentrations ($p \leq 0.05$)

24 h concentrations of 13.3 µmol/mg protein and 11.8 µmol/mg protein, respectively. Conversely, the concentration of thiols in bacteria exposed to the highest AAA concentration (10 mM) significantly decreased from the first sampling (10 µmol/mg protein) until the end of the assay (7.9 µmol/mg protein).

Discussion

Regulation of the *uspA* and *dhaT* genes by *L. reuteri* in response to AAA

L. reuteri counts remained stable with the increasing applied doses of AAA (1 mM, 5 mM, and 10 mM) during the entire experimental assay (37 °C/24 h), so the survival was not jeopardized (data not shown). Yet, the challenge with this oxidized amino acid led to impairments of the bacterium's physiology. This finding reflects the ability of *L. reuteri* to activate mechanisms to neutralize the potential harmful effects of the sub-lethal concentrations of the added oxidized amino acid. In the present study, these mechanisms were firstly assessed by the analysis of the relative expression of stress-related genes.

The universal stress protein A (UspA) superfamily includes an ancient and conserved group of proteins found in assorted microorganisms, insects, and plants. The precise roles of Usp proteins in biological systems remain unclear; yet, they seem to be involved in the defense against DNA-damaging agents and respiratory uncouplers (Kvint et al. 2003). Due to the defined function of the gene *uspA*, an upregulation was expected, which was only observed at 24 h and in the presence of the highest AAA concentration (Fig. 1a). Yet, the downregulation observed at earlier

samplings and lower concentrations is consistent with data reported by Oberg et al. (2015), who found a significant downregulation of the *uspA* gene expression in *Bifidobacterium longum* exposed to a hydroxyl-radical generating system. Similar results were reported by Arcanjo et al. (2019) working on the same bacterium and strain from the present study. In that study, exposing *L. reuteri* to 0.5 mM of hydrogen peroxide led to a significant decrease of the *uspA* gene expression. It is worth noting that both aforementioned studies found the occurrence of oxidative stress and molecular damage in the exposed bacteria. The fact that AAA exposure led to a similar effect on *L. reuteri* indicates that this oxidized amino acid is identified by the bacterium as a chemical threat. In fact, two recent studies agree in describing noxious effects of food-compatible AAA concentrations (200 μM) on human intestinal (Díaz-Velasco et al. 2020) and human acinar pancreatic cells (Estaras et al. 2020). According to these authors, the harmful effect of AAA involved the induction of pro-oxidative conditions within cells. Probiotic bacteria like *L. reuteri* may also be susceptible to this chemical species and, according to these results, the downregulation of *the uspA* gene seems to be related to a cellular signal of a pro-oxidative threat that both, the radical generating systems (i.e., hydrogen peroxide) and oxidized amino acids such as AAA, may be able to induce.

It is worth clarifying that the higher AAA concentrations tested in the present study (1–10 mM) are plausibly compatible with a physiological situation as explained as follows. While AAA concentration in foods has been found to reach up to 200 μM, it is also known that dietary proteins are further oxidized during digestion, increasing significantly the final concentration of oxidized amino acids in the gut. For instance, in a study by Van-Hecke et al. (2019), the concentration of protein oxidation products increased between 2 and fivefold times in assorted foods after simulated gastrointestinal digestion. The same authors found in a more recent study (Goethals et al. 2020) sixfold times higher concentrations of protein oxidation products in pork digests than in the original (undigested) pork product.

The *dhaT* gene encodes the enzyme 1,3-PDO oxidoreductase which is known to play a relevant role in stressful situations involving energetic demand. This enzyme enables the main carbohydrate fermentation pathway (6-phosphogluconate/phosphoketolase; 6-PG/PK) through the production of NAD^+ (required for glucose fermentation) from NADH in the conversion of 3-hydroxypropionaldehyde (3-HPA) (its substrate) into 1,3-PDO under anaerobic conditions. Additionally, 3-HPA, also known as reuterin, is excreted by *L. reuteri* strains under stressful situations (Schaefer et al. 2010). The overexpression of this gene observed in bacteria exposed to 5 and 1 mM AAA for 20 and 24 h, respectively, could respond to an attempt of the bacteria to protect against the oxidative threat caused by this oxidized amino acid. The elemental mechanisms by which *L. reuteri* may seek to protect against AAA-induced biological damage through the activation of the 3-HPA pathway should be subjected to scrutiny. As previously reported by Talarico et al. (1988), the 3-HPA pathway requires glycerol, commonly added as growth promoter in *Lactobacillus* cultures. In the present study, *L. reuteri* had no access to such precursor, and, therefore, the 3-HPA pathway is unlikely to have occurred. Considering the absence of glycerol, it seems reasonable to consider that 1,3-PDO may have other substrates and that its cellular activity may be related to protection against a potential pro-oxidative threat. To similar conclusions came Arcanjo et al. (2019) who found an increased expression of the *dhaT* gene in *L. reuteri* challenged with hydrogen peroxide in simulated colonic conditions where glycerol was, again, absent. The authors hypothesized whether the NAD + -dependent activity of the 1,3-PDO may be able to detoxify hydrogen peroxide in the presence of NADH. Since no hydrogen peroxide was included in the present assay, the implication of 1,3-PDO in balancing the redox state of the cell seems to be a pertinent defense mechanism against pro-oxidative threats. It is, still unknown how AAA may impair the redox status of *L reuteri* but it is proven that AAA exposure to human eukaryotic cells cause oxidative stress via mitochondrial disturbance and ROS generation (Díaz-Velasco et al. 2020; Estaras et al. 2020).

It is worth noting that the effect of AAA exposure on the expression of the *dhaT* gene at early stages of the assay (12 and 16 h) was opposite to that observed at advanced stages. As discussed in due course, the activation of the gene at advanced stages of oxidative stress and oxidative damage could have triggered defense mechanisms, in which the *dhaT* gene may be implicated. At early stages, the underexpression of this gene could respond to indefinite initial responses of the bacteria to the AAA exposure, in which the protein encoded by this gene was not found as essential. In line with this downregulation, a recent study by Díaz-Velasco et al. (unpublished data) observed that AAA exposure to CACO-2 cells led to an overall downregulation of gene expression due to the impairment of protein kinase A and C (PKA and PKC, respectively) signaling pathways. Yet, the mechanisms implicated in the downregulation of *dhaT* gene at early stages of exposure to AAA in this bacterium remain indefinite and require further elucidation.

ROS generation in *L. reuteri* by AAA

The increased production of ROS in *L. reuteri* by the presence of AAA has no precedent in literature (Fig. 2). It is, however, consistent with results reported by Díaz-Velasco et al. (2020) in CACO-2 cells and Estaras et al. (2020) in pancreatic cells when the exposure to AAA led to impairment of the oxidative status of the cell, ROS generation,

apoptosis, and necrosis. In addition, it is in accordance to Da Silva et al. (2017) who studied the effect of AAA on brain function of adolescent rats, and showed an induction of ROS generation and alteration of the cellular redox status via mitochondrial impairment. While the percentage of *CelRox* positive bacteria was found to be relatively low, previous studies using hydrogen peroxide and malondialdehyde (MDA) as inductors of oxidative stress in *L. reuteri* reported similar percentages (Arcanjo et al. 2019; Padilla et al. 2021). The oxidative damage caused in bacterial lipids and proteins, explained in due course, denote severe oxidative stress. The precise mechanisms by which AAA is able to induce ROS generation in *L. reuteri* are indefinite. It is worth noting that such mechanisms differ from those reported by the aforementioned authors since the bacterium lacks mitochondria. Interestingly, *Lactobacillus spp.* have also been found to be able to produce hydrogen peroxide and other ROS via implication of NAD(P)H oxidoreductases (Hertzberger et al. 2014) which provides a plausible and coherent connection between AAA exposure, *dhaT* overexpression and ROS generation. The molecular mechanisms underlying the interconnection between all these elements need to be precisely described.

Oxidative damage to *L. reuteri* by AAA

In the present work, the oxidative damage to bacterium caused by AAA-induced oxidative stress was assessed by means of TBARS (lipid oxidation) and allysine (protein oxidation). The basal TBARS concentration in control cultures, (~ 1 mg/L) may correspond to the occurrence of lipid peroxidation in the bacterium under physiological conditions and did not change significantly during the assay within groups (Fig. 3a). AAA did not significantly affect the extent of lipid oxidation in *L. reuteri.*

On the other hand, AAA exposure had a significant impact on the oxidative damage to bacterial proteins. A relatively low but significant increase in allysine, the main protein carbonyl in biological systems (Stadtman and Levine 2000; Estévez and Luna 2016), was observed in the control group of *L. reuteri* (Fig. 3b). The present results show that allysine, formed in bacteria, as in eukaryotes, remarkably contributes to protein carbonylation and may be used as a reliable indicator of oxidative stress. The results obtained are in accordance with Ezraty et al. (2017) who proposed that protein carbonylation could be a reflection of bacterial senescence as oxidized proteins accumulate in non-proliferating bacteria. Allysine is typically formed in proteins because of the attack of ROS to lysine residues. This is plausibly the mechanism taking place in the present assay as the significant production of ROS in *L. reuteri* exposed to AAA exposure could have caused the oxidation of lysine residues and hence, the accretion of allysine. Once formed, allysine may also react with amino groups from neighboring amino acids (e.g., lysine) to form an azomethine structure, also known as Schiff bases (Estévez 2011). The dramatic drop of allysine concentration during the last 4 h of the assay in the bacterium exposed to the highest concentration of AAA (10 mM) is consistent with the sudden increase of Schiff bases in that period of time (Fig. 3c). These results suggest that such fluorescent structures were, at least, partially formed in bacteria exposed to 10 mM as a result of allysine addition to other protein amines. The formation of Schiff bases in bacteria exposed to intermediate AAA doses (1 and 5 mM), was not so intense to reflect a decline of the reactant (allysine). Both, carbonylation and formation of non-reducible protein cross-links (i.e., Schiff bases), are irreversible protein modifications with negative biological consequences (Davies 2005; Ezraty et al. 2017; Estévez and Xiong 2019). Carbonylated proteins can be dysfunctional and may be labeled to removal due to its accumulation causes impaired homeostasis that leads to chronic dysfunction and apoptosis (Shacter 2003). However, carbonylated proteins can also act as signaling molecules, which may activate specific pathways, to preserve homeostasis control senescence (Shacter 2003).

Both situations could be applied to the present experiment. The increase in carbonyls above 10 nmol/mg proteins in the bacterium challenged with 5 and 1 mM of AAA was coincident with the activation of the *dhaT* gene at sampling times of 20 h and 24 h, respectively, and plausibly, the corresponding synthesis of the NADH-dependent oxidoreductase decoded by this gene. Given the proposed role of this enzyme in detoxifying pro-oxidant species (Arcanjo et al. 2019), a relatively mild pro-oxidative threat, exhibited in a significant accretion of protein carbonyls, could have led to the activation of an antioxidant response mediated, among others, by the activation of the *dhaT* gene. On the other hand, a severe oxidative damage caused by a more intense pro-oxidative environment, such as that observed in *L. reuteri* challenged with the highest concentration of AAA (10 mM) led to a sudden formation of advanced oxidation products (Schiff bases) and no *dhaT* gene-mediated response against the oxidative insult. These mechanisms were not present in the bacterium incubated with the lowest doses of AAA (1 and 5 mM). Previous considerations made by Ezraty et al. (2017) and Arcanjo et al. (2019) support the hypothesis that the *dhaT* gene could have been activated by pro-oxidant species and/or the effect of the former on protein carbonylation.

The evolution of protein thiols during the assay (Fig. 4) provides additional strength to the aforementioned hypotheses. The oxidation of sulfur-containing amino acids, such as cysteine (Cys) and methionine (Met), is a typical feature in biological systems attacked by ROS (Estévez et al. 2020). While the oxidation of thiols in proteins may lead to dysfunction, irrelevant sulfur-containing amino acids are known to act as antioxidants offering a sacrificial loss to ROS and

protecting other amino acids with relevant significance, such as lysine (Davies, 2005; Estévez et al. 2020). This dual role of thiols was examined in the present experiment. Taking into account that these moieties can act as redox-active compounds and elements of antioxidant protection in biological systems, the coincidence of thiol accretion with the increase of carbonylation in those samples may respond to a strategy to keep a balanced redox status in cells in danger. The incubation of *L. reuteri* with AAA caused an increase of thiol concentration since 12 h incubation onwards. The pro-oxidant changes induced by AAA, including the formation of protein carbonyls, possibly triggered the accumulation of thiol groups by the novo synthesis of sulfur-containing proteins/peptides with the purpose of protecting the bacterium against this pro-oxidant threat. Thiol accumulation is considered as an endogenous mechanism of antioxidant defense owing to the recognized redox-active properties (Davies 2005). These moieties have been typically regarded as elements of antioxidant protection in eukaryotes and in lactic acid bacteria (Schaefer et al. 2010; Xiao et al. 2011). However, the molecular mechanism backing the synthesis of thiol-containing species remain unclear and needs further clarification. It is worth noting that such thiol accretion did not take place in cultures treated with 10 mM of AAA, confirming the lack of genetic (*dhaT* mediated) and antioxidant response in these bacteria. The irreversible loss of thiols in this group of bacteria may respond to the consumption of these moieties in the severe pro-oxidative environment caused by 10 mM of AAA.

Conclusions

The present results show, for the first time, that a food-occurring oxidized amino acid, the AAA, is able to disturb the redox balance of the probiotic bacterium *L. reuteri* by inducing the formation of ROS and causes protein oxidative damage. This bacterium seems to be able to activate both genetic and molecular mechanisms to struggle with the oxidative threat. The *dhaT* gene is proposed to play a role by encoding a NAD + -dependent oxidoreductase that may contribute to detoxify oxidizing species. The specific effects exerted by the highest AAA concentrations are more unlikely to be occur in physiological conditions while the exact amount of free AAA in food digests is yet to be defined. Finally, the present results and their consequences for the microbiota and the impact on the host may be further studied in upcoming in vivo studies.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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4.IV. Chlorogenic acid modulates the antioxidant response of *Enterococcus faecium* to oxidative stress: A flow cytometry and proteomic study

El ácido clorogénico modula la respuesta antioxidante de *Enterococcus faecium* al estrés oxidativo: un estudio proteómico y de citometría de flujo.

Abstract

Certain phytochemicals have been found to promote the beneficial effects of probiotic bacteria although the molecular mechanisms of such interactions are poorly understood. The objective of the present study was to evaluate the impact of the exposure to 0.5 mM chlorogenic acid (CA) on the redox status and proteome of *Enterococcus faecium* isolated from cheese and challenged with 2.5 mM hydrogen peroxide (H_2O_2). The bacterium was incubated in anaerobic conditions for 48 h at 37 ºC. CA exposure led to a more intense increased oxidative stress and accretion of bacterial protein carbonyls than those induced by H_2O_2. The oxidative damage to bacterial proteins was even more severe in the bacterium treated with both CA and H_2O_2, yet, such combination led to an strengthening of the antioxidant defenses, namely, a catalase-like activity. The proteomic study indicated that H_2O_2 caused a decrease in energy supply and the bacterium responded by reinforcing the membrane and wall structures and counteracting the redox and pH imbalance. CA stimulated the accretion of proteins related to translation and transcription regulators and hydrolases. This phytochemical was able to counteract certain proteomic changes induced by H_2O_2 (i.e. increase of ATP binding cassete (ABC) transporter complex) and cause the increase of Rex, a redox-sensitive protein implicated in controlling metabolism and responses to oxidative stress.

Keywords: *E. faecium;* chlorogenic acid; oxidative stress; proteomics; flow cytometry.

4. RESULTADOS

1. INTRODUCTION

Oxidative stress is involved in a variety of pathological conditions including cardiometabolic and neurological disorders, diabetes, and chronic inflammatory diseases, among others (Dos Santos et al., 2019). Some of them, such as the inflammatory bowel diseases (IBD) (Bourgonje et al., 2020) and colorectal carcinomas (Chen et al., 2019), are located in the gastrointestinal tract (GIT). While the etiology of these pathologies is diverse, the pathophysiological mechanisms typically involve persistent oxidative stress and chronic inflammation (Alemany-Cosme et al., 2021; Bourgonje et al., 2020). Scientific evidence supports the potential benefits of dietary interventions in alleviating the oxidative stress occurred in the lumen and tissues of the GIT (Ballini et al., 2019; Sugihara et al., 2019). Among these antioxidant strategies, the intake and colonization of probiotic bacteria (Ballini et al., 2019) and certain dietary phytochemicals (Mastrogiovanni et al., 2019) have been identified as effective in protecting against benign intestinal inflammatory processes.

The beneficial impact of probiotic bacteria from *Lactobacillus* and *Bifidobacterium* genera on gut health is profusely documented (Jakubczyk et al., 2020). Other bacteria such as certain species and strains from *Enterococcus* have been much less studied for their potential health benefits. Strains of *Enterococcus faecium*, isolated from fermented food products such as cheeses, have been recently characterized for their biosafety and probiotic potential (Oruc et al., 2021). In addition to their biopreservation effects in fermented foods by the synthesis and excretion of bioactive peptides, *Enterococcus faecium* is able to colonize the GIT and exert diverse physiological benefits (Choeisoongnern et al., 2021). Working on an *in vivo* mice model, Divyashri et al. (2015) observed antioxidant, anti-inflammatory and neuromodulatory effects of oral supplements of ***E. faecium*** and ***Lactobacillus rhamnosus***. More recently, Huang et al. (2021) were able to prevent obesity and hyperlipidemia in C57BL/6 mice by dietary administration of *E. faecium* R0026 together with *Bacillus subtilis* R0179. In both cases, the authors emphasized the necessity of pinpointing the molecular mechanisms behind these probiotic effects.

Phytochemicals with assorted bioactivities such as antioxidant, antimicrobial, anti-inflammatory, antidiabetic and antiproliferative, among others, have also attracted considerable attention for their potential health benefits (Shahidi and Ambigaipalan, 2015). Chlorogenic acid (CA), a bioactive component of assorted plant materials and foods such as coffee and apples, has been profusely studied for its antioxidant, anti-inflammatory (Farah and Lima, 2019), antihypertensive (Loader et al., 2017) and antiglycemic effects (Wang et al., 2008) and may contribute to protect against

cardiovascular, metabolic, cancerogenic and neurological disorders (Tajik et al., 2017). Interestingly, CA has been described to act as prebiotic and hence, enable the occurrence of functional properties exerted by probiotic bacteria. In the study carried out by Palócz et al. (2016), the combination of CA with *Lactobacillus plantarum* 2142 alleviated intestinal inflammation and oxidative stress in IPEC-J2 cells. Certain species of bifidobacteria and other gut microbiota are known to biotransform CA into bioactive compounds that are speculated to exert some of the aforementioned health benefits (Raimondi et al., 2015; Tomás-Barberán et al., 2014). Yet, the underlying molecular mechanisms of the interactions between CA and probiotic bacteria are not well understood.

The present study aims to decipher the molecular basis of the interaction between CA and ***E. faecium*** isolated from matured cheese, in a simulated anaerobic and pro-oxidant colonic environment. To achieve this objective, the bacteria were exposed to dietary concentrations of CA (0.5 mM) for 48 h and subsequently analyzed by means of high-resolution mass spectrometry-based proteomics using a Nanoliquid Chromatography-Orbitrap MS/MS. Supportive flow cytometry studies and protein oxidation measurements were applied to assess the impact of the pro-oxidant environment and CA on the bacteria.

2. MATERIAL AND METHODS

2. 1. Chemicals and raw material

All chemicals and reagents used in this study were of ACS analytical grade and purchased from Sigma Chemicals (Sigma-Aldrich, USA), Scharlab S.L. (Spain), Pronadisa (Conda Laboratory, Spain), Applied Biosystems (USA), Epicentre (USA) and Acros Organics (Spain). *E. faecium* was isolated from matured soft cheese and identified by 16S rRNA gene sequencing (Ordiales et al., 2013). This identification was further confirmed by the proteomic results.

2.2. Experimental settings

Stock cultures of *E. faecium* were stored at -80 °C in Man, Rogosa and Sharpe (MRS) broth / glycerol to a final concentration of 20% (v/v). Before experimental use, *E. faecium* was subcultured twice under anaerobic conditions at 37 °C for 24 h in MRS broth supplemented with 0.5% L-Cysteine (m/v). Four experimental groups were considered depending on the chemical challenges applied to the bacteria: CONTROL (*E. faecium* in MRS), H_2O_2 (*E. faecium* in MRS + 2.5 mM H_2O_2), CA (*E. faecium* in MRS + 0.5 mM CA) and H_2O_2 + CA (*E. faecium* in MRS + 2.5 mM H_2O_2 + 0.5 mM CA). Before its incorporation in the corresponding cultures, CA was dissolved in dimethyl sulfoxide (DMSO) to facilitate solubility. The

volume of DMSO used to dissolve and deliver the CA to treated cultures (CA and H_2O_2 + CA) was also added to the other groups (CONTROL and H_2O_2). Three replicates were carried out for each treatment. Experimental groups were incubated at 37 °C for up to 48 h in anaerobic conditions. Samples of the cultures were collected in four times (0, 12, 24 and 48 h) from the inoculation. For further analyses, culture medium was removed by washing with a phosphate buffered saline solution (PBS, pH 7.4) twice. For counting of viable cells, 100 µL of *E. faecium* were inoculated on MRS agar at the same sampling time and conditions as already reported for the experimental groups.

2.3. Analytical procedures

2.3.1. Reactive oxygen species (ROS) generation by flow-cytometry analyses

Flow cytometry detection of ROS in *E. faecium* was carried out according to the previous described protocol (Padilla et al., 2021). Briefly, *E. faecium* ($1x10^6$ cfu/mL)) sampled at 48 h was suspended in 1 mL of PBS and stained with Hoechst 33342 (0.5 µM) (Sigma, Steinheim, Germany) to discriminate bacterium from debris and CellRox Deep Red (5 µM) (ThermoFisher, CA, USA) for the detection of intracellular ROS. Excitation and emission wavelengths were set at 345 nm and 488 nm, respectively, for the Hoechst 33342, and at 644 and 645nm, respectively, for the CellRox Deep Red. Prior to analyses, the samples were filtered through MACS® SmartStrainer 30 µm filters and immediately run on a Cytoflex® flow cytometer (Beckman Coulter, CA, USA) equipped with violet, blue and red lasers. The instrument was daily calibrated using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. However due to emission and excitation characteristics of the combination of probes used, spectral overlap was negligible. Files were exported as FCS files and analyzed using FlowjoV 10.5.3 Software for Mac OS (Ashland, OR, USA). Unstained, single-stained and Fluorescence Minus One (FMO) controls were used to determine compensations and positive and negative events, as well as to set regions of interest.

2.3.2. Quantification of hydrogen peroxide (H_2O_2)

H_2O_2 was quantified in *E. faecium* by using the procedure described by Jiang et al. (1990) with some modifications. Briefly, *E. faecium* ($1x10^6$ cfu/mL) sampled at 0, 12, 24 and 48h were diluted in hexane/isopropanol (3:1, v/v). The mixture was vigorously vortexed and subsequently dispensed in quartz cuvettes to measure absorbance at 240 nm in an 1800 Shimadzu spectrophotometer (SHIMADZU EUROPA GmbH, Duisburg, Germany). Standard curves were prepared with standard H_2O_2

for quantification purposes using the extinction coefficient of 43.6 M^{-1} * cm^{-1} at 240 nm. Data is expressed as pmol of H_2O_2/L.

2.3.3. Assessment of catalase-like activity

The ability of *E. faecium* to decompose H_2O_2 was assessed by the procedure reported by Li and Schellhorn (2007) with some modifications. Briefly, *E. faecium* ($1x10^6$ cfu/mL) sampled at 48h was exposed to a 20 mM H_2O_2 solution and allowed to stand at room temperature (22 ºC) for 180 s. Each 30 s, the depletion of H_2O_2 was assessed by measuring absorbance at 240 nm in an 1800 Shimadzu spectrophotometer (SHIMADZU EUROPA GmbH, Duisburg, Germany). Standard curves were prepared with standard H_2O_2 for quantification purposes using the extinction coefficient of 43.6 M^{-1} * cm^{-1}. Data was expressed as pmol of depleted H_2O_2/min*mL.

2.3.4. Quantification of bacterial protein carbonyls

Total protein carbonyls were determined in *E. faecium* sampled at 48 h by means of the dinitrophenylhydrazine (DNPH) method as described by Estévez et al. (2019) with minor modifications. Briefly, proteins from *E. faecium* ($1x10^6$ cfu/mL) were precipitated by the addition of 1 mL of cold 10% trichloroacetic acid (TCA), followed by centrifugation at 4 °C at 600 g for 5 min. The supernatants were discarded and protein pellets were treated with 1 mL of a 2 M HCl solution with 0.2% DNPH and incubated at room temperature for 1 h. Proteins were subsequently precipitated with 1 mL of cold 10% TCA, followed by centrifugation at 4 °C, 1200 g for 10 min and washed twice with 1 mL of ethanol:ethyl acetate (1: 1 v/v). The pellets were dissolved in 1.5 mL of 20 mM Na_3PO_4 buffer pH 6.5 added with guanidine hydrochloride to reach 6 M. The amount of carbonyls was expressed in nmoles of protein hydrazones per mg of protein using a molar extinction coefficient of hydrazones of 21.0 nM^{-1} cm^{-1} at 370 nm.

2.3.5. Quantification of bacterial TBARS

Malondialdehyde (MDA) and other TBARS were quantified in each *E. faecium* culture, sampled at 0, 12, 24 and 48 h by adding 200 µL of the bacterial suspension to 500 µL thiobarbituric acid (0.02 M) and 500 µL trichloroacetic acid (10%). Samples were incubated during 20 min at 90 °C. After cooling, a 5 min centrifugation at 600 g was made and the supernatant was measured at 532 nm. Results are expressed as mg TBARS per L of sample.

2.3.6. Sample preparation for LC-MS/MS Based Proteomics

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Sample preparation for proteomics was carried out with 200 mL of culture of each experimental group sampled at 48 h with three biological replicates per treatment. Cells were collected by centrifugation at 4 °C, 6000 g for 10 min, supernatant was discarded, and the pellet was resuspended in 4 mL lysis buffer pH 7,5 (100 mM Tris-HCl, 50 mM NaCl, 10% glycerol, 20 mM EDTA pH 8,5), 1 mM PMSF (Phenylmethansulfonylfluorid) and 1 µg/mL Pepstatin A. The collected cells were subjected to rupture by pressure difference using a French press with approximately 68 atm. The resulting lysate was placed at 4 °C for 1 h and then centrifuged at 4 °C, 12,000 g, for 15 min to remove cell debris and residual intact cells. The clarified supernatants containing the majority of proteome of *E. faecium* were precipitated with trichloroacetic acid/acetone (Carpentier et al., 2005).

The protein precipitated lysates were resuspended in 8M urea. Protein concentration was measured with a Coomasie Protein Assay Reagent Ready to Use employing a Nanodrop 2000c Spectophotometer and a Nanodrop 2000 software (USA) to ensure homogeneous protein quantities in the different samples. Five aliquots per treatment, three biological replicates plus two technical replicates, containing 50 µg of proteins were treated as previously described by Delgado et al. (2015a, b) and Owens et al. (2015). Briefly, samples were incubated with 0.5 M DTT (Dithiothreitol) in 50 mM ammonium bicarbonate for 20 min at 56 °C for protein reduction. The resulting free thiol (-SH) groups were alkylated by incubating the samples with 0.55 M iodoacetamide in 50 mM ammonium bicarbonate for 15 min at room temperature in the dark. Sequencing-grade trypsin (Promega, USA) and ProteaseMAX surfactant (Promega, USA) were added and incubated at 50 °C for 1 h. Finally, 1 µL of 100% formic acid was added to stop the proteolysis reaction. Supernatant was removed and placed into new screw-capped Eppendorf tubes for drying in a vacuum concentrator. Digested samples were then desalted, prior to spectrometric analysis, using Pierce™ C18 Tips (Thermo Scientific, USA).

Before analyzing the samples on the Orbitrap LC-MS/MS, digested samples were resuspended in loading buffer (98% milli-Q water, 2% acetonitrile, 0.05% trifluoroacetic acid), and sonicated in a water bath for 5 min and centrifuged at 14.452 g for 15 min at room temperature and put them into vials for LC-MS/MS Orbitrap.

2.3.7. Label-free quantitative (LFQ) proteomic analyses

A Q-Exactive Plus mass spectrometer coupled to a Dionex Ultimate 3000 RSLCnano (Thermo Scientific, USA) analyzed around 2 µg from each digest. Data was collected using a Top15 method for MS/MS scans (Delgado et al., 2019). Comparative proteome abundance and data analysis were carried out using MaxQuant software (v. 1.6.0.15.0;

https://www.maxquant.org/download_asset/maxquant/latest) and Perseus (v 1.6.14.0) to organize the data and perform statistical analysis. Carbamidomethylation of cysteines was set as a fixed modification; oxidation of methionines and acetylation of N-terminals were set as variable modifications. Database searching was performed against *E. faecium* protein database (www.uniprot.org). The maximum peptide/protein false discovery rates (FDR) were set to 1% based on comparison to a reverse database. The LFQ algorithm was used to generate normalized spectral intensities and infer relative protein abundance. Proteins were identified with at least two peptides, and those proteins that matched to a contaminant database or the reverse database were removed, and proteins were only retained in final analysis if they were detected in at least two replicates from at least one treatment. Quantitative analysis was performed using a t-test to compare treatments with the CONTROL. The qualitative analysis was also performed to detect proteins that were found in at least three replicates of a given treated group but were undetectable in the comparison CONTROL group. All proteins satisfying one of the two aforementioned criteria are identified as "discriminating proteins".

2.4. Statistical Analysis

All experiments were performed five times (3 biological replicates + 2 technical replicates) and each individual sample was analyzed twice for flow cytometry. Data was analyzed for normality and homoscedasticity. The effect of the exposure to H_2O_2 and AC was assessed by Analysis of Variance (ANOVA). The Tukey's test was used for multiple comparisons of the means. The effect of the incubation time on the same measurements was assessed by Student's t-test. The significance level was set at $p < 0.05$. SPSS (v. 15.0) was used for statistical analysis of the data.

3. RESULTS & DISCUSSION

3.1. Onset of oxidative stress in *E. faecium* exposed to H_2O_2 and CA

After the incubation of *E. faecium* under the tested experimental conditions, the counts for the four experimental groups were similar, reaching the stationary phase in 12 h (Figure 1). While H_2O_2 did not compromise the survival of *E. faecium* at the applied concentrations, the achievement of the stationary phase in bacteria treated with H_2O_2 (H_2O_2 and H_2O_2+CA), was retarded (Figure 1). This is in agreement with previous works that tested the effect of H_2O_2 on *Lactobacillus reuteri* and other probiotic bacteria and observed a growth delay along with oxidative damage in bacterial proteins and lipids via ROS formation (Arcanjo et al., 2019; Sanders et al., 2004). The presence of H_2O_2 drives to an oxidative

environment through the generation of free radicals producing damage to lipids and proteins. On the other hand, the presence of CA alleviated the negative effect that H_2O_2 had on the growth of the bacteria (Figure 1). These results agree with a previous work where the presence of resveratrol acted as a protector of the bacteria against oxidative stress (Arcanjo et al., 2019). Other authors have also encountered a positive effect of selected phenolic compounds on the survival, growth and REDOX status of probiotic bacteria at the expense of pathogenic microorganisms, since phenolic compounds have the ability to inhibit oxidative stress by acting as metal chelators and radicals scavengers (Cardona et al., 2013; Estévez and Heinonen, 2010).

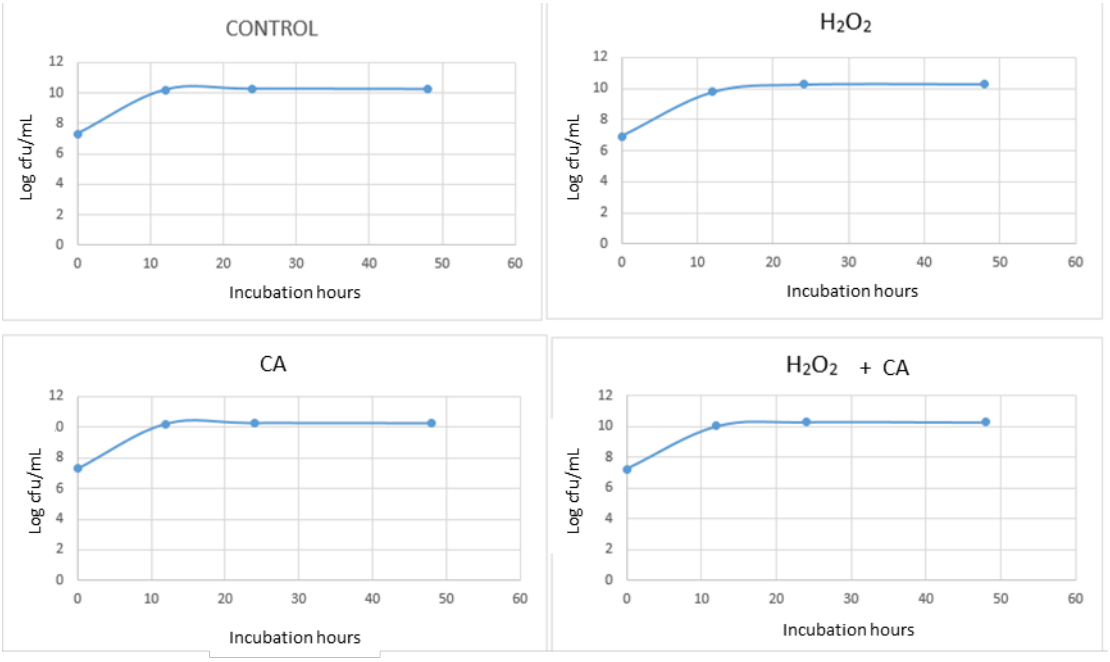

Figure 1. Evolution of the growth of *Enterococcus faecium* during its incubation in the presence of H_2O_2, chlorogenic acid (CA) and the combination of both (H_2O_2 + CA) compared to CONTROL group.

lAmong the techniques to measure oxidative stress in bacteria, flow cytometry was used to analyze the population of CelRox + bacteria (%) (Figure 2A) and the total fluorescence emitted by ROS in *E. faecium* (Figure 2B) at the end of the incubation period (48 h).

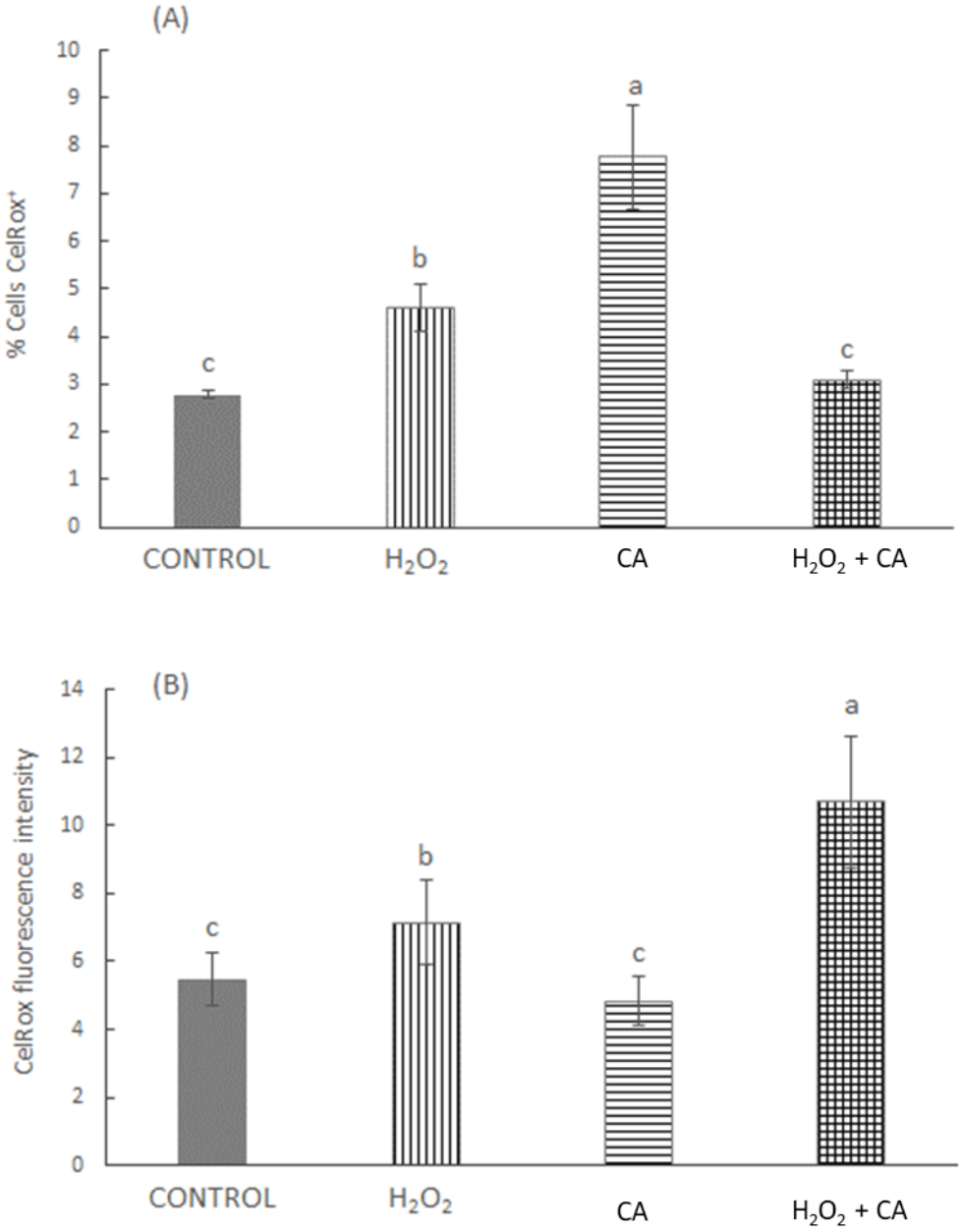

Figure 2. Subpopulation of *Enterococcus faecium* (%) phenotyped by Reactive oxygen species (ROS) occurrence (Cell Rox +; A), and total fluorescence emitted by ROS in *E. faecium* (B). Results expressed as means ± standard deviations. Different letters on top of bars denote significant differences ($p < 0.05$) between group of samples.

This technique showed that the bacterial populations are distributed into several groups depending on the amount of CelRox probe bound to the ROS present in these cells: CelRox+ bacteria with a significant amount of ROS and CelRox - bacteria that may have an indeterminate amount of ROS, but it is not enough to be detected. The results displayed significant differences between treatments,

which prove that the presence of both H_2O_2 and CA, and their combination, affected the generation of free radicals and their concentration in *E. faecium*. As expected, H_2O_2 stimulated the formation of free radicals in bacteria (H_2O_2 *vs* CONTROL). The decomposition of H_2O_2 into the hydroxyl radical through the Fenton reaction with a transition metal is well described in the literature and is represented below:

$$M^n + H_2O_2 \rightarrow M^{n+1} + HO^{\bullet} + HO^{-}$$

Where M is a transition metal, HO− is the hydroxyl ion and HO • is the hydroxyl radical, the most abundant and dangerous free radical in biological systems (Davies, 2005). Despite a transition metal was not included, the medium has many of them that could have catalyzed the decomposition of H_2O_2. Fluorescence emitted by the probe specifically binds to ROS present in the cells and its intensity is directly related to their amount of ROS. Therefore, the incubation with H_2O_2 not only increased the number of bacteria with significant ROS concentration, but the total amount of ROS in these samples was higher than those of the CONTROL.

The results in the bacterium treated with CA did not find coherence between the population of CelRox+ (Figure 2A) and the total amount of ROS (Figure 2B). On the one hand, CA stimulated the formation of ROS in *E. faecium* in such way that the population of CelRox+ was significantly higher than those incubated with the pro-oxidant H_2O_2. These results could be considered contradictory because of the antioxidant effect attributed to this phenolic compound (Sato et al., 2011). Nevertheless, it is also well documented the ability of CA to act as a pro-oxidant and to stimulate the formation of ROS in biological systems (Hou et al., 2017). Specifically, the increase in ROS formation by CA has been observed in human cancer cells (CACO-2) and non-transformed epithelial cells cultured *in vitro* (Yan et al., 2015). Furthermore, a recent study performed with flow cytometry in *Bacillus subtilis* and *Escherichia coli* showed that their incubation with CA induced high levels of ROS, which resulted in oxidative damage to the bacteria (Wang et al., 2020). However, previous research examined the formation of ROS and the survival of *E. coli* incubated with CA finding a reduction in the ROS content (Lee and Lee, 2018), which is opposite to the results obtained in the present study. Nevertheless, the mechanisms involved in the generation of ROS by CA are still to be thoroughly defined and may be affected by many factors such as the concentrations of CA and H_2O_2, among others. In previous works carried out *in vitro*, it was proposed that the pro-oxidant effects of CA were exerted by its oxidized form (quinone) (Utrera and Estévez, 2012). The effect of CA increased the population of CelRox+ (Figure 2A) with total fluorescence levels lower than those found in cells incubated with H_2O_2 and similar to those of the CONTROL group (Figure 2B). These results reflect that the amount of ROS in the population of CelRox+ bacterium and,

in general, in the groups treated with CA, is much lower than those treated with H_2O_2. In other words, CA was able to induce oxidative stress, slightly but significantly, in a higher number of the bacterium than the H_2O_2. The mechanisms of ROS generation by CA, still unknown, seem to be different from those of H_2O_2 and may respond to more complex cellular mechanisms than the simple decomposition of a reactive molecule, as occurs with H_2O_2.

An opposite result was observed when the bacterium was exposed to the combination of H_2O_2 and CA. The population of CelRox+ bacteria treated with both substances was similar to the CONTROL group (Figure 2A). Nevertheless, total fluorescence levels (Figure 2B) were significantly higher than the other groups under study. Therefore, cells exposed to H_2O_2 and CA had, on average, the highest levels of radicals, but they were concentrated in a small population of the bacterium (similar to the CONTROL group). Compared to the effect of H_2O_2, alone, the combination with CA decreases the number of CelRox+ bacteria, which proves a protective effect of the phenolic compound against the propagation of oxidative stress. However, bacterium undergoing stress and CelRox+, have higher ROS levels than those from other groups of the bacterium.

3.2. Oxidative damage in lipids and proteins from *E. faecium* exposed to H_2O_2 and CA

Figure 3 shows the oxidative damage to lipids (Figure 3A) and proteins (Figure 3B) from *E. faecium* after 48 h of incubation. The concentration of MDA was consistent with the generation of free radicals. Incubation with H_2O_2 caused an increase in ROS content that led, in turn, to oxidative damage to the cell lipids. The addition of CA, in the presence and in the absence of H_2O_2, led to a significant reduction in MDA levels. The protective effect of this phenolic compound against lipid oxidation could have been caused via the induction of slight oxidative stress (observed in the flow cytometry analysis), that would, in turn, have caused a reinforcement of the cell antioxidant defences, (as observed in the catalase-like activity analysis, discussed in the following section). Protection against lipid oxidation is essential since lipids of biological membranes constitute (with the cell wall) the first and most important barrier of the cell and its interaction with the environment. The results found in this work are consistent with previous works in which the addition of a phenolic compound increases the oxidative stability of the lipids of probiotic bacteria incubated in a free radical generating system (Arcanjo et al., 2019).

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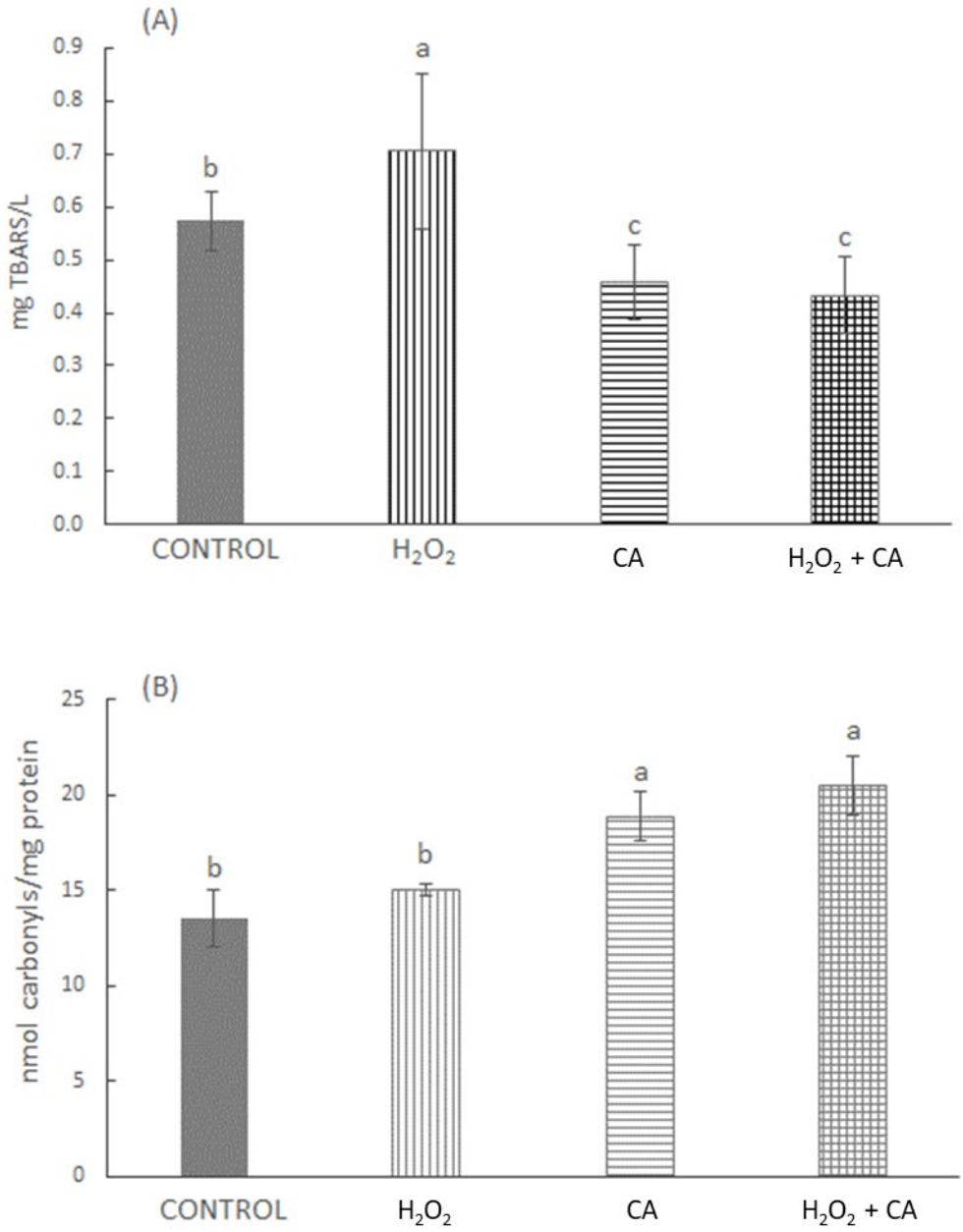

Figure 3. Oxidative damage to lipids (A) and proteins (B) from *Enterococcus faecium* as analyzed by the concentration of thiobarbituric reactive substances (TBARS) and concentration of protein carbonyls, respectively. Results expressed as means ± standard deviations. Different letters on top of bars denote significant differences ($p < 0.05$) between group of samples.

The results of protein oxidation were different than those obtained from lipid oxidation, which highlights that the oxidation of lipids and proteins respond to different biological mechanisms. Unlike lipid oxidation, protein oxidation has been described as a mechanism of cell regulation and signalling in bacteria and eukaryotic cells (Arcanjo et al., 2019; Ezraty et al., 2017). The addition of CA, alone or

in combination with H_2O_2, caused a remarkable increase of the protein carbonyls. The pro-oxidant effect of CA on bacterial proteins could respond to the well-known pro-oxidant mechanism of its oxidized quinone form (Utrera and Estévez, 2012). As already proposed by Ezraty et al. (2017) and Arcanjo et al. (2019), protein carbonylation could be a cell signalling mechanism that trigger the strengthening of endogen antioxidant defence systems. This hypothesis would explain the increase in protein carbonylation and the protection against cell lipids. In the present study, such strengthening effect on the antioxidant defences would include, among others, an increased ability of the bacterium to degrade H_2O_2 (catalase-like activity, discussed in the following section). In agreement with the present results, Arcanjo et al. (2019) observed how the stimulation of carbonyl formation in *L. reuteri* by resveratrol occurred along with an upregulation of genes encoding for enzymes with antioxidant activity which, ultimately, protected bacterial lipids against oxidation. These authors also proposed that the protection of resveratrol against oxidative stress in *L. reuteri* would have made via strengthening the antioxidant defences of the cell at the expense of stimulating protein oxidation. This latter oxidative damage may act as a sign of a pro-oxidative threat and the triggering event for the strengthening the antioxidant defences via gene expression.

3.3. Catalase-like activity in *E. faecium* exposed to H_2O_2 and CA

The ability of *E. faecium* to detoxify H_2O_2 after 48 h of incubation is shown in Figure 4. Such probiotic bacterium is Gram positive and catalase negative, condition that can make it susceptible to suffer oxidative stress in the presence of H_2O_2. Catalase enzyme decomposes two molecules of this reactive species into molecules of water and molecular oxygen according to the following reaction:

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

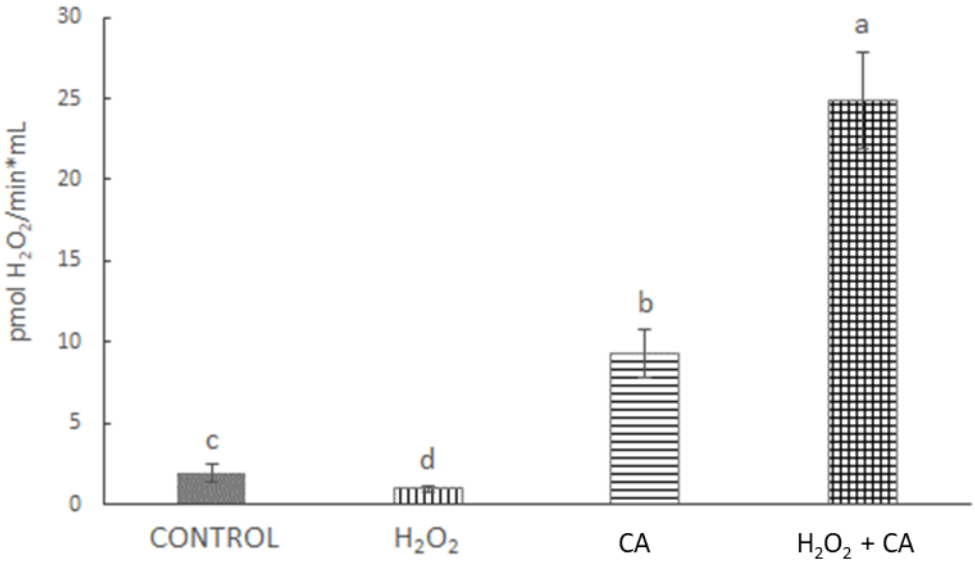

Figure 4. Catalase-like activity of *Enterococcus faecium*as analyzed by its ability to decompose H_2O_2. Results expressed as means ± standard deviations. Different letters on top of bars denote significant differences ($p < 0.05$) between group of samples.

However, it is also known that some lactic acid bacteria possess an activity called catalase-like, which usually depends on an NADH-dependent oxidoreductase enzyme (Tanaka et al., 2018). This characteristic could respond not only to an antioxidant protection mechanism and REDOX balance control, but also to a protection mechanism against other bacteria and energy metabolism regulation. In a previous work, the *dhaT* gene, which encodes for an NADH-dependent oxidoreductase enzyme, was observed to be overexpressed in bacteria *L. reuteri* exposed to oxidative stress induced by H_2O_2 (Arcanjo et al., 2019). According to the authors, the enzyme would be able to detoxify H_2O_2. Kang et al. (2013) have also reported a similar enzyme in *Lactobacillus panis*.

In this work, *E. faecium* showed a catalase-like activity, which differed between experimental units (Figure 4). The catalase-like activity of the CONTROL group was significantly reduced in the presence of H_2O_2. However, incubation with CA increased the catalase-like activity by 5-fold in these bacteria. The pathway by which the phenolic compound causes an increase in this antioxidant activity, still unknown, is considerably enhanced by the co-exposure to H_2O_2. This result contributes to explain the protective effect of CA against lipid oxidation in *E. faecium* caused by H_2O_2 (described above) and reinforces the hypothesis that the mechanism may involve the oxidized form of CA. In this regard, it seems reasonable to hypothesize that CA (or more likely its oxidized quinone form) causes a mild oxidative stress in cells (measured by flow cytometry and confirmed by protein carbonylation) that

triggers the catalase-like activity described above. Thus, H_2O_2, which itself causes oxidative stress without activating the antioxidant defences, would contribute to oxidize CA, which in turn would cause a considerable increase in catalase-like activity. This hypothesis is in accordance with the findings from Arcanjo et al., (2019) in a test carried out *in vitro* where *L. reuteri* was incubated in the presence of H_2O_2 and resveratrol. These authors demonstrated that the gene *dhaT*, which encodes the enzyme 1,3-propanediol oxidoreductase (1,3-PDO), is overexpressed in a situation of oxidative stress and that resveratrol has regulatory effect on such expression. The authors proposed that it would be the oxidized form of resveratrol which could be behind the signalling mechanism that would trigger the expression of the gene and the activation of this antioxidant defence. In the present research, an increase in catalase-like activity is observed that could respond to the presence of a similar enzyme, such as the one described by Tanaka et al. (2018).

3.4. Proteome response of *E. faecium* by the exposure to H_2O_2 and CA

The LFQ proteomic analysis carried out revealed a total of 1041 proteins in at least three out of the five replicas in at least one of the four treatments (CONTROL, CA, H_2O_2 and CA + H_2O_2). Each one of these proteins was identified by at least two peptides and its FDR was lower than 1%. (Supplementary Tables 1, 2 and 3).

3.4.1. E. faecium proteome response to H_2O_2 exposure.

Among the proteins significantly modified in quantity ($p < 0.05$) in *E. faecium* because of the exposure to H_2O_2, 77 proteins were found in lower quantity and 211 were in higher quantity as compared to the CONTROL. Attending to the qualitative analysis, one single protein was found only in the bacterium when treated with H_2O_2, and another was detected only in the CONTROL group (Supplementary Table 1). These proteins were identified as discriminating proteins as their relative concentration was significantly affected by the exposure to H_2O_2 (Table 1).

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Table 1. Discriminating proteins from *Enterococcus faecium* as affected by the exposure to 2.5 mM H_2O_2 for 48 h at 37 ºC and anaerobiosis.

H_2O_2 vs CONTROL DOWN					
PROTEIN NAME	GENE NAME	*p*-value	Fold-change[1]	BIOLOGICAL FUNCTION	FASTA accession number
Biomolecule synthesis proteins					
Serine hydroxymethyltransferase	*glyA*	0.00452454	0.85984171	Amino-acid biosynthesis	I3U4H4
Leucine--tRNA ligase	*leuS*	0.0375858	0.93509873	Protein biosynthesis	Q3Y3J5
Pyrroline-5-carboxylate reductase	*proC*	0.00355311	0.85845594	Amino-acid biosynthesis,	Q3XYJ7
Serine--tRNA ligase	*serS*	0.0460292	0.92275528	Protein biosynthesis	I3U1P9
Cysteine synthase	*cysK2*	0.0117679	0.84167432	Amino-acid biosynthesis	I3TZB4
4-hydroxy-tetrahydrodipicolinate synthase	*dapA*	0.00355311	0.85845594	Amino-acid biosynthesis	Q3Y1B7
Pyrroline-5-carboxylate reductase	*proC*	0.00452454	0.85984171	Amino-acid biosynthesis	I3U4H4
Glycolytic Process					
Enolase	*eno*	0.0268079	0.86465409	glycolytic process	Q3Y0T4
Fructose-bisphosphate aldolase	*fba2*	0.00616979	0.78393612	glycolytic process	I3U007
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	*gpmA*	0.00266733	0.86230073	Gluconeogenesis, Glycolysis	Q3XXS6
Triosephosphate isomerase	*tpiA*	5.37E-05	0.84181435	Gluconeogenesis, glycolysis	Q3XX07
L-lactate dehydrogenase	*ldh*	0.00554119	0.84350177	glycolytic process	Q3XWM4
Glucose-6-phosphate isomerase	*pgi*	0.0094401	0.82259716	Gluconeogenesis, glycolysis	Q3XZ32
Pyruvate oxidase	*poxB*	0.0274256	0.89857947	pyruvate oxidase activity	Q3XZD0
H_2O_2 *vs* CONTROL UP					
Peptidoglycan Biosynthesis					
Lipid II isoglutaminyl synthase (glutamine-hydrolyzing) subunit GatD	*cobQ*	0.027	1.17368479	Peptidoglycan synthesis	Q3Y1G9
UDP-N-acetylglucosamine 1-carboxyvinyltransferase	*murAB*	0.0207546	1.23346861	Peptidoglycan synthesis	I3U008
UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--L-lysine ligase	*murE*	0.0194958	1.26894628	Peptidoglycan synthesis	Q3XXY2
UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide)	*murG*	9.24E-05	1.235876	Peptidoglycan synthesis	Q3Y2H8

pyrophosphoryl-undecaprenol N-acetylglucosamine transferase					
Endolytic murein transglycosylase	*pabC*	0.0194958	1.26894628	Cell wall biogenesis/degradation	Q3XXY2
Acyl carrier protein	*acpP2*	0.0186913	1.40619337	Fatty acid biosynthesis	Q3Y0S6
3-oxoacyl-[acyl-carrier-protein] synthase 3	*fabH*	0.0114654	1.34891552	Fatty acid biosynthesis	Q3Y0S7
Transcription					
DNA-directed RNA polymerase subunit alpha	*ntd*	0.0476046	1.53248293	transcription	Q3Y0A5
Histidine kinase	*vicK*	0.00153634	1.24927665	Two-component regulatory system	Q3XYG9
Histidine kinase	*HMPREF0351_12688*	0.00159151	1.21516566	Two-component regulatory system	Q3XYJ6
Chaperone protein ClpB	*clpB*	5.96E-05	1.57368305	Stress response	Q3XZF2
Protein RecA	*recA*	9.79E-08	1.79455607	DNA damage, DNA recombination, DNA repair, SOS response	Q3Y1B9
DNA replication and repair protein RecF	*recF*	0.0474494	1.34561435	DNA damage, DNA replication, DNA repair, SOS response	Q3XZE3
DNA gyrase subunit A	*gyrA*	0.0118261	1.09394257	DNA-dependent DNA replication, DNA topological change	Q3XZE1
Transcriptional regulator	*HMPREF0351_10139*	0.0122266	2.63167257		I3TYC5
LysR family transcriptional regulator	*HMPREF0351_10567*	0.00470219	1.2419723		I3TZK3
Response regulator	*HMPREF0351_12687*	0.028874	1.11573159	Transcription, Transcription regulation	I3U5M3
DeoR family transcriptional regulator	*fruR*	0.0129794	1.40397281	Transcription, Transcription regulation	I3U2J9
Heat-inducible transcription repressor HrcA	*hrcA*	2.96E-05	1.57250761	Stress response, transcription, transcription regulation	Q3XWX7
Lactose PTS family porter repressor	*lacR*	0.00277818	1.27691191	Transcription, Transcription regulation	Q3XYZ6
Transcription-repair-coupling factor	*mfd*	0.00343941	1.14442586	DNA damage, DNA repair	Q3Y182
DNA-binding response regulator	*vicR*	0.00164261	1.15741962	Transcription, Transcription regulation	I3U4P8
Chromosomal replication initiator protein DnaA	*dnaA*	0.0114346	1.09805247	DNA replication	Q3XZE6
Endonuclease MutS2	*mutS*	0.0217157	1.20521372	mismatch repair, negative regulation of DNA recombination	Q3XXL7
Endonuclease MutS2	*mutS3*	0.00391418	1.2182515	mismatch repair, negative regulation of DNA recombination	Q3Y1S0

ATPases					
GTPase Obg	*cgtA*	0.00710349	1.15336648	ribosome biogenesis	Q3XWL6
ATP-binding subunit of chaperone	*HMPREF0351_10960*	0.000153	1.42978379	ATPase activity, ATP binding	Q3Y0T6
p-ATPase superfamily P-type ATPase heavy metal transporter	*HMPREF0351_10990*	0.00419889	1.81844967	ATP binding, hydrolase activity	Q3Y0Q6
p-ATPase superfamily cation transporter	*HMPREF0351_11086*	0.00040997	1.29297197	ATPase activity, ATP binding	I3U122
p-ATPase superfamily P-type ATPase copper (Cu) transporter	*copB*	0.017299	1.33412237	ATP binding	I3U3B6
DNA repair protein RadA	*radA*	0.00655028	1.28103421	DNA damage, DNA repair, stress response	I3U5E2
Teichoic acid ABC superfamily ATP binding cassette transporter, ABC protein	*tagH*	0.0139462	1.24166931	ATPase-coupled transmembrane transporter activity	Q3XY30
p-ATPase superfamily cation transporter	*yoaB*	0.00018857	1.21270025		I3U105
ABC transporter					
ABC superfamily ATP binding cassette transporter, ABC protein	*HMPREF0351_10042*	0.00127376	1.19032126		Q3XXS0
ABC superfamily ATP binding cassette transporter, ABC protein	*HMPREF0351_10435*	0.00054375	1.31671034		Q3Y2U7
ABC superfamily ATP binding cassette transporter, ABC protein	*HMPREF0351_10455*	0.0446083	1.19724798	ATPase-coupled transmembrane transporter activity, ATP binding, hydrolase activity	Q3Y2W7
ABC superfamily ATP binding cassette transporter, ABC protein	*HMPREF0351_10571*	0.0034535	1.37607299	ATPase-coupled transmembrane transporter activity, ATP binding, hydrolase activity	I3TZK7
ABC superfamily ATP binding cassette transporter, ABC protein	*HMPREF0351_11163*	0.0136578	1.2564219	ATPase-coupled transmembrane transporter activity, ATP binding	Q3XWK0
ABC superfamily ATP binding cassette transporter, membrane protein	*HMPREF0351_12246*	0.0451943	1.21250021	ATPase-coupled transmembrane transporter activity, ATP binding	I3U4D2
ABC superfamily ATP binding cassette transporter, membrane protein	*HMPREF0351_12247*	0.00014756	1.32115249	ABC-type amino acid transporter activity, ATPase-coupled transmembrane	Q3XWY8

				transporter activity. ATP binding, hydrolase activity	
Glutamate ABC superfamily ATP binding cassette transporter, ABC protein	*glnQ*	0.045557	1.1956963	ABC-type amino acid transporter activity, ATPase-coupled transmembrane transporter activity. ATP binding, hydrolase activity	Q3XZW4
Phosphotransferase System					
HPr kinase/phosphorylase	*hprK*	0.038432	1.17618989	Carbohydrate metabolism	Q3XWV5
Phosphoenolpyruvate-protein phosphotransferase	*proS*	0.00368226	1.07864117	Phosphotransferase system, sugar transport	Q3XZ69
Serine/Threonine kinases					
Non-specific serine/threonine protein kinase	*HMPREF0351_12565*	0.00797501	1.15164814	Kinase, Serine/threonine-protein kinase, Transferase	Q3Y195
HPr kinase/phosphorylase	*hprK*	0.038432	1.17618989	Carbohydrate metabolism	Q3XWV5

[1]C: only found in CONTROL samples. T: only found in treated samples.

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According to ClueGO classification, the proteins found in lower quantity belonged to several metabolic routes, namely glycolytic processes (53.70%), carboxylic acid metabolic processes (18.52%) and exopeptidase activities (16.67%). Non-mentioned pathways accounted for less than the 10% (Supplementary Figure 1A). While this is, to our knowledge, the first time that bacterial proteome is studied in relation to the impact of H_2O_2, our results confirm those from some other previous studies. For instance, the lower relative abundance of proteins involved in glycolytic processes matches with those results from Ranjbar et al. (2020) who indicated the disruption of the glycolytic pathway in *E. coli* in a pro-oxidative environment (such as that made by H_2O_2) that could explain, in turn, a reduced bacterial growth (Sanman et al., 2016). Likewise, Zhang et al. (2021) found a down-regulation of nucleotide biosynthesis in *Pediococcus pentosaceus* R1 exposed to H_2O_2, which is consistent with the lower abundance of proteins involved in carboxylic acid metabolic process and exopeptidase activity, found in the present study (Table 1). Unlike the previous studies, the present work indicates how the exposure to H_2O_2 affected the concentration of specific proteins playing a relevant role in essential bacterial processes such as energy supply and biomolecules synthesis (Table 1), providing, like this, a more complete and accurate picture of the impairment of biological processes in the bacterium challenged by a pro-oxidative challenge.

On the other hand, the increased routes were related to organic substance biosynthetic processes (48%), anion binding (30%) and peptidoglycan biosynthesis (10%). Non-mentioned pathways accounted for less than the 10% (Supplementary Figure 1B). Therefore, the exposure to H_2O_2 led to an increase of proteins involved in DNA and proteins synthesis and transcription regulators, ATP binding cassete (ABC) transporters, oxidoreductases, DNA repair proteins and peptidoglycan biosynthesis (Table 1). These results agree with other research studying lactic acid bacteria adaptation responses and proteins related to nutritional adaptation (hydrolases, ABC transporters and phosphotransferase systems (PTS)), and stress mechanisms, such as membrane modification and anti-oxidative defences, were found (Lebeer et al., 2011). According to our results, *E. faecium* would be activating routes related to protect biologically relevant biomolecules (proteins, DNA and peptidoglycan) and recover the physiological impairments caused by H_2O_2 in terms of energy supply, redox status and pH. The possible function of ATPases found in anion binding route could be to ensure the pH homeostasis, as seen by Arena et al. (2006). This could be because *E. faecium* was incubated in anaerobiosis, and therefore fermentation of the glucose present in the media was carried out, giving a lower pH, and activating some proteins found in acid environment stress, as ATPases. This situation may be worsened in the presence of H_2O_2 owing to the connection between oxidative stress, altered redox status and impaired pH (Tsai et al., 1997). This relation has been described in eukaryotic cells

(Tsai et al., 1997) and human tissues (Loh et al., 2002). Nevertheless, as far as we know, it is the first time that it has been detailed in bacteria These findings could support the idea that bacterial adaptation to oxidative stress may involve the contribution of different physiological mechanisms (Arena et al., 2006).

The exposure of *E. faecium* to H_2O_2 also caused an increased in vicK and another protein (HMPREF0351_12688) encoding two histidine kinases that are part of the two-component system (Table 1).The two-component systems are mechanisms that the bacterium utilize to respond to environmental changes and are composed by a sensor histidine kinase that autophosphorylate and subsequently transfer the phosphate group to their cognate response regulators thus modulating their activity, usually as transcriptional regulators (Monedero et al., 2017). Consistently, Zhou et al. (2010) found an upregulation of this gene *in Desulfovibrio vulgaris* Hildenborough incubated with H_2O_2. These proteins seem to be involved in the detection and response to oxidative stress as regulators.

Serine/Threonine kinases were also found in higher quantity in *E. faecium* treated with H_2O_2 (Table 1). These enzymes regulate protein activity through its association with phosphatases, to quench signalling cascades (Pereira et al., 2011) and it is well known that these proteins are involved in stress responses (Sasková et al., 2007).

The *cgtA* gene, also named *Obg*, encodes for an essential GTPase that plays a role in adjust the cellular energy status, control DNA replication, ribosome biogenesis and stress adaptation pathways (stringent response, sporulation, general stress response) (Kint et al., 2014) (Table 1). Nevertheless, these proteins seem to exert its effect specifically through the regulator RecA, also found in *E. faecium*, which is involved in regulation of homologous recombination on top of its function in promoting the auto-proteolysis of LexA, repressor of SOS regulon (Cox, 2007). This means that cgtA and RecA are entailed in stress response. Interestingly, we have identified the clpB protein which has been recently described in *E. faecium* as part of a stress induced multi-chaperone system, in cooperation with dnaK, dnaJ and GrpE. This system is involved in the recovery of the cell from heat stress and in the correct folding of newly formed proteins (Alam et al., 2021). Yet no significant differences were found between treatments.

The enzyme alkyl hydroperoxide reductase (ahpC), which is part of AhpF-AhpC complex, provides *Streptococcus mutans* with peroxidase activity (Higuchi et al., 2000), catalyzing the reduction of peroxide into water or alcohol. AhpC is also involved in a chaperone function, as found in *Helicobacter pylori,* stabilizing the protein folding in oxidative stress conditions (Chuang et al., 2006). Although no

significative differences were found among treatments for AphC, the relative abundance of this proteins in the CONTROL group was the lowest, indicating that chemical challenges would have stimulated the synthesis of this protective protein.

Peptidoglycan biosynthesis has been considered as a vital strategy for lactic acid bacteria to cope with various stress condition (Yang et al., 2021) (Table 1). The exposure to H_2O_2 stimulated the occurrence of proteins related to cell wall and membrane synthesis, encoded in *E. faecium* by genes such as *acp* (Acyl carrier protein) and *fab* (3-oxoacyl-[acyl-carrier-protein] synthase 3). This mechanism was also found in *L. plantarum* ZDY2013 in response to an acid challenge (Huang et al., 2016). These higher quantity in cell wall proteins may contribute to maintaining cell functions of the bacteria under stress condition (Yang et al., 2021).

Finally, Gls20 (general stress protein) was found in 1.53 and 1.17-fold higher quantities in H_2O_2 and CA groups, respectively (Table 1). Both groups showed higher ROS levels than the CONTROL treatment (Section 3.1). This protein has been described to be essential for multiple stress adaptation in *E. faecalis* (Giard et al., 2001; Teng et al., 2005) and for oxidative stress in *S. thermophilus* (Arena et al., 2006).

3.4.2 *E. faecium* proteome response to CA exposure.

Among the proteins significantly modified in quantity ($p < 0.05$) in *E. faecium* because of the exposure to CA, 98 were in lower quantity and 119 were in higher quantity, as compared to the CONTROL. In the qualitative analysis just four proteins were found only in the CA treatment (Supplementary Table 2)

Based on the ClueGO classification, the proteins found in lower quantity belonged to several metabolic routes, namely to carboxylic acid metabolic processes (30.3%), to anion binding processes (30.3%) and to organonitrogen compound metabolic processes (24.2%) (Supplementary Figure 2A). Basically, the proteins found in these routes are related to biomolecules synthesis, including subpathways, such as protein, lipids, and fatty acid biosynthesis (Table 2). This finding is coherent with our previous results, as under the CA treatment the bacterium is under stress and in this situation, the bacterial response is linked to a slower growth. To similar conclusions came Arena et al. (2006) who found that exposure of *S. thermophilus* to oxidative stress led to inhibition of bacterial growth.

On the other hand, the increased routes were related to translation; including oxidoreductases, transcription regulators, ribosomal proteins and amino acid synthesis proteins (58%); and to anion binding, where hydrolases and transcription regulators, among others, were identified (42%) (Table 2).

Pathways accounting for less than the 10% of total metabolic routes affected by CA, were not enumerated (Supplementary Figure 2B).

Table 2. Discriminating proteins from *E. faecium* as affected by the exposure to, 0.5 mM chlorogenic acid (CA) for 48 h at 37 ºC and anaerobiosis.

CA *vs* CONTROL DOWN					
PROTEIN NAME	GENE NAME	*p*-value	Fold-change[1]	BIOLOGICAL FUNCTION	FASTA accession number
Biomolecule Synthesis					
Arginine--tRNA ligase	*argS*	0.0111687	0.83851471	Protein biosynthesis	Q3XZA3
Asparagine--tRNA ligase	*asnS*	0.00141641	0.89032378	Protein biosynthesis	Q3XYR6
Aminotransferase	*aspB*	0.0194228	0.76697369	biosynthetic process	Q3XYR7
Aspartate aminotransferase	*aspC2*	0.0335775	0.90835239	biosynthetic process	I3U1B3
Aspartate--tRNA ligase	*aspS*	0.0458164	0.90679667	Protein biosynthesis	Q3XY17
Glycine--tRNA ligase alpha subunit	*glyQ*	0.00078655	0.77646618	Protein biosynthesis	I3U3K9
Glycerol-3-phosphate dehydrogenase [NAD(P)+]	*gpsA*	0.0145494	0.68762552	Lipid biosynthesis	Q3XWV3
GMP synthase [glutamine-hydrolyzing]	*guaA*	0.00426934	0.90352411	Purine biosynthesis	Q3XZ01
Elongation factor 4	*lepA*	0.00289396	0.85116516	Protein biosynthesis	I3U2C9
Leucine--tRNA ligase	*leuS*	0.00839025	0.92249692	Protein biosynthesis	Q3Y3J5
Enoyl-[acyl-carrier-protein] reductase [NADH]	*fabI*	0.00066351	0.8273261	Fatty acid biosynthesis, Fatty acid metabolism, Lipid biosynthesis, Lipid metabolism	I3U1N1
5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	*mtnN*	0.0414773	0.7878943	Amino-acid biosynthesis, Methionine biosynthesis	Q3Y2D9
CA vs CONTROL UP					
Transcription Regulator					
Protein RecA	*recA*	0.0140556	1.09030064	DNA damage, DNA recombination, DNA repair, SOS response	Q3Y1B9
Global transcriptional regulator Spx	*spxA2*	0.0118128	1.12167988	Transcription, Transcription regulation	Q3XYC5
RNA-binding heat shock protein	*HMPREF0351_12550*	0.0376856	1.5011665	Stress response	Q3Y180
Bifunctional ligase/repressor BirA	*birA*	0.0373255	1.13330957	Transcription, Transcription regulation	I3U5K7
Chromosomal replication initiator protein DnaA	*dnaA*	0.0003706	1.08299456	DNA replication	Q3XZE6

Replicative DNA helicase	*dnaB*	0.00831094	1.15824383	DNA replication	Q3XZD5
ATP synthase subunit beta	*atpD*	0.0305479	1.12829084	ATP synthesis, Hydrogen ion transport, Ion transport, Transport	Q3XY89
GTPase Obg	*cgtA*	0.00796315	1.18855775	ribosome biogenesis	Q3XWL6
Endonuclease MutS2	*mutS3*	0.0112118	1.17828293	mismatch repair, negative regulation of DNA recombination	Q3Y1S0
Bifunctional ligase/repressor BirA	*lplA*	3.43E-05	1.15246585	Transcription regulation	Q3XZT0
Crp family transcriptional regulator	*crp*	0.0483219	1.21978003	Transcription regulation	Q3XZ99
DeoR family transcriptional regulator	*fruR*	0.00515452	1.43849878	Transcription regulation	I3U2J9
Transcription termination/antitermination protein NusA	*nusA*	0.0308972	1.17973841	Transcription regulation	Q3XZ66
Biomolecule Synthesis					
Cysteine--tRNA ligase	*cysS*	0.0255754	1.1420549	Protein biosynthesis	Q3Y163
Glutamate--tRNA ligase	*gltX*	0.00431243	1.14860202	Protein biosynthesis	Q3Y161
Histidine--tRNA ligase	*hisS*	0.0470706	1.07215984	Protein biosynthesis	Q3XY18
Serine--tRNA ligase	*serS*	0.00258078	1.146762	Protein biosynthesis	Q3XYJ7
50S ribosomal subunit assembly factor BipA	*tufA*	0.00047291	1.14600155	Protein biosynthesis, Ribosome biogenesis	I3TY41
Elongation factor Tu	*tufA2*	0.0021493	1.16160454	Protein biosynthesis	Q3XX23
Valine--tRNA ligase	*valS*	0.0250311	1.09358549	Protein biosynthesis	Q3Y009
Chorismate synthase	*aroC*	0.00805999	1.24290239	Amino-acid biosynthesis, Aromatic amino acid biosynthesis	Q3XXZ3
Glutamate--tRNA ligase	*gltX*	0.00431243	1.14860202	Protein biosynthesis	Q3Y161
Translation initiation factor IF-3	*infC*	0.0325331	1.1527215	Protein biosynthesis	I3TYL6
Ribosomal Proteins					
50S ribosomal protein L1	*rplA*	0.00082174	1.24556905	Translation regulation	I3U4S7
Ribosomal protein L2	*rplB2*	0.0249151	1.14152306	translation	Q3XYY6
50S ribosomal protein L3	*rplC*	0.00135762	1.22568648	translation	I3TY43
50S ribosomal protein L6	*rplF*	0.00429825	1.29648544	translation	Q3XYX4
50S ribosomal protein L13	*rplM*	0.0267301	1.143132	translation	Q3Y121

50S ribosomal protein L15	*rplO*	0.0452591	1.1521272	translation	Q3XYX0
50S ribosomal protein L17	*rplQ*	0.0334508	1.13329386	translation	Q3XYW2
50S ribosomal protein L18	*rplR*	0.00618316	1.33207656	translation	Q3XYX3
50S ribosomal protein L22	*rplV*	0.0366711	1.19631308	translation	Q3XYY4
50S ribosomal protein L30	*rpmD*	0.0139636	1.35832339	translation	Q3XYX1
50S ribosomal protein L33	*rpmG*	0.038802	1.46801021	translation	I3U2V2
50S ribosomal protein L35	*rpmI*	0.0001304	1.26171931	translation	Q3XXD4
30S ribosomal protein S3	*rpsC*	0.00111061	1.29653756	translation	Q3XYY3
30S ribosomal protein S4	*rpsD*	0.0062631	1.16890364	translation	I3TYB5
30S ribosomal protein S5	*rpsE*	0.0173987	1.12529714	translation	Q3XYX2
30S ribosomal protein S10	*rpsJ*	0.0126307	1.1348794	translation	I3TY42
30S ribosomal protein S11	*rpsK*	0.0394981	1.27752542	translation	Q3XYW4
30S ribosomal protein S12	*rpsL*	0.00264341	1.40648484	translation	I3TY38
30S ribosomal protein S14	*rpsN2*	0.00637147	1.25672762	translation	Q3XWD4
30S ribosomal protein S17	*rpsQ*	0.00335901	1.20269765	translation	Q3XYY0
30S ribosomal protein S19	*rpsS*	0.0201825	1.13995115	translation	Q3XYY5
30S ribosomal protein S21	*rpsU*	0.0157863	1.19428073	translation	Q3Y1C1
Oxidoreductase					
GMP reductase	*guaC*	0.00129811	1.14720165	purine nucleotide metabolic process	Q3XYY6
Glyceraldehyde-3-phosphate dehydrogenase	*gap*	0.0323958	1.14555125	glucose metabolic process	I3TY43

The increase in proteins acting as transcription regulators could be related to the pro-oxidant activity of CA, which may trigger a signalling cascade to activate the antioxidant defences of the cell, as observed in the catalase-like activity of this group. According to Lushchak (2011), the activation of the antioxidant responses follows several steps that includes sensing reactive species and pass the signal through regulators to transcription and translation machineries. As a major transcriptional regulator described in the response to oxidative stress (Kajfasz et al., 2012), we identified SpxA2 (Table 2) that was found in 1.12- and 1.11-fold higher quantities when exposing the bacterium to CA and H_2O_2 + CA, respectively, than in CONTROL bacteria. This protein could be triggered by the oxidative stress caused by the quinone form of the chlorogenic acid and therefore cause a signal that could provoke the proposed catalase-like activity discussed above. The mechanism of action of Spx protein involves the interaction with the C-terminal domain of the RNA polymerase α-subunit as demonstrated *in vitro* and is thus, a modulator of the transcriptional regulator – RNA polymerase interaction (Zuber, 2004). In the *Streptococcaceae* family, SpxA2 maintain activation of oxidative stress genes and in addition is involved in cell envelope homeostasis (Nilsson et al., 2019). The plausible implication of CA in promoting protein carbonylation would incriminate this post-translational change as the sensing mechanism that would eventually trigger the antioxidant response as proposed by Ezraty et al. (2017).

Among the translation proteins, an important number of ribosomal proteins were in higher quantity in *E. faecium*, with these proteins having the ability to build the ribosomal small and large units. Furthermore, they display extra-ribosomal functions, as being a part of an operon, whose activity could modulate, in final steps, even stress responses (Singh et al., 2009). Ribosomal proteins (RplJ, RpsF) were found to be increased in *L. sakei* as a response to stress caused by high hydrostatic pressure (Jofré et al., 2007). These family of proteins are plausibly related to the response of *E. faecium* towards the induced stress caused by CA.

Finally, in the anion binding route, we discovered several hydrolases that could be involved in the degradation pathway of the CA, because one of the steps of the transformation of this compound is the hydrolysis (Tomas-Barberan et al., 2014). In *Bifidobacterium animalis*, a feruloyl esterase activity capable of hydrolysing CA was identified (Raimondi et al., 2015) and although this enzyme was not found in *E. faecium*, one of these hydrolases could have this function.

3.4.3 *E. faecium* proteome response to H_2O_2 + CA exposure.

Considering the initial hypothesis that CA may counteract the potential negative effects of H_2O_2 in *E. faecium*, the effect of H_2O_2 + CA exposure on the proteome is compared to that exerted by just H_2O_2.

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Among the proteins significantly modified in quantity ($p < 0.05$) in *E. faecium* because of the exposure to H_2O_2 + CA, 47 were in lower quantity and 65 were in higher quantity, as compared to the bacteria exposed to H_2O_2. In the qualitative analysis, five proteins were found only in the H_2O_2 treatment (Supplementary Table 3).

Based on the ClueGO classification, the simultaneous addition of CA + H_2O_2 to bacteria, led to a decrease in quantity of proteins involved in just one metabolic route, namely to the ABC transporter complex (100%) (Supplementary Figure 3A). It is worth recalling that this group of proteins was also increased in bacteria treated with H_2O_2 as compared to the CONTROL bacteria. Therefore, CA seems to activate this particular route, counteracting the increase caused by the H_2O_2.

On the other hand, the increased routes were related to organonitrogen compound biosynthetic process (92%) and to other processes not displayed for accounting less than 5% of the total routes affected by the exposure to CA (Supplementary Figure 3B) (Table 3). Among those found in greater quantity, the main pathway included proteins associated with ribosomal proteins and transcriptional regulators such as the abovementioned spxA2.

Table 3. Discriminating proteins from *E. faecium* as affected by the exposure to 2.5 mM H_2O_2, and 0.5 mM chlorogenic acid (CA) for 48 h at 37 ºC and anaerobiosis.

H_2O_2+CA *vs* H_2O_2UP					
PROTEIN NAME	GENE NAME	*p*-value	Fold-change[1]	BIOLOGICAL FUNCTION	FASTA accession number
ABC transporter					
Amino acid ABC superfamily ATP binding cassette transporter, membrane protein	*HMPREF0351_10153*	0.0138275	0.86273656	amino acid transport	I3TYD9
Amino acid ABC superfamily ATP binding cassette transporter, membrane protein	*HMPREF0351_12278*		H	Amino-acid transport, Transport	I3U4G4
Glycine betaine/carnitine/choline ABC superfamily ATP binding cassette transporter, membrane/binding protein	*proWX*		H	Amino-acid transport, Transport	Q3Y0D9
Redox-sensing transcriptional repressor Rex	*rex*	0.0383895		Transcription, transcription regulation	Q3Y0H7
H_2O_2+CA *vs* H_2O_2DOWN					
Ribosomal protein					
50S ribosomal protein L6	*rplF*	0.0309578	1.28499523	translation	Q3XYX4
50S ribosomal protein L9	*rplI*	0.0226826	1.12089023	translation	Q3XZD6
50S ribosomal protein L13	*rplM*	0.0384216	1.10405267	translation	Q3Y121
50S ribosomal protein L19	rplS	0.00981996	1.23072207	translation	I3U367
50S ribosomal protein L33	rpmG	0.00154458	1.84346241	translation	I3U2V2
50S ribosomal protein L35	rpmI	0.00567683	1.19665975	translation	Q3XXD4
30S ribosomal protein S7	rpsG	0.0247056	1.1207465	translation	I3TY39
30S ribosomal protein S10	rpsJ	0.0410132	1.14588638	translation	I3TY42
30S ribosomal protein S12	rpsL	0.00367784	1.44583408	translation	I3TY38
30S ribosomal protein S17	rpsQ	0.00029301	1.16493947	translation	Q3XYY0

Transcription regulation					
RNA polymerase sigma factor SigA	sigA	0.0282087	1.23233543	Transcription, Transcription regulation	Q3XWZ6
Global transcriptional regulator Spx	spxA2	0.0381649	1.09836077	Transcription, Transcription regulation	Q3XYC5
Bifunctional ligase/repressor BirA	lplA	0.0307981	1.09852599	Transcription, Transcription regulation	Q3XZT0
GTP-sensing transcriptional pleiotropic repressor CodY	codY	0.0449831	1.13376764	Transcription, Transcription regulation	I3U178

Finally, a transcriptional repressor, Rex, had significant differences, 1.09-fold higher quantity. This protein is a regulator that respond to cellular NAD^+/NADH ratio in order to modulate gene expression in central metabolism, oxidative stress response and biofilm formation (Vesić and Kristich, 2013). In this study *E. faecalis* has shown that Rex factor influences the production or detoxification of H_2O_2, similar effect could have in *E. faecium* being an important protein for H_2O_2 elimination. Yet, further studies are required to clarify the connection between the Rex protein and the strengthening of the antioxidant defences in the bacterium exposed to CA. Likewise, further studies are required to identify the enzymes involved in the catalase-like activity of *E. faecium*.

5. Conclusions

This paper originally shows that CA displays a protecting effect on probiotic *E. faecium* via modulating its proteome and strengthening its endogenous antioxidant defences. These results and the hypotheses proposed here as in line with modern theories proposing that i) the overall protective effect of certain phytochemicals on the redox status of cells is not made through their radical scavenging activities but actually via inducing a mild oxidative stress that stimulates their own antioxidant protection means and that ii) protein carbonylation likely induced by the oxidized forms of such phytochemicals in a pro-oxidative environment, would play a role as a signalling mechanisms that would trigger the antioxidant strengthening mechanisms. This protection may be proved *in vivo* conditions to guarantee that such effects lead to benefits in experimental animals or human volunteers affected by disorders in which oxidative stress plays a major role.

Credit authorship contribution statement

P. Padilla: Data curation, Methodology, Formal analysis, Writing - original draft.

M.J. Andrade: Data curation, Methodology, Funding acquisition, Supervision, Formal analysis, Validation, Writing – review & editing.

J. Delgado: Data curation, Methodology, Funding acquisition, Supervision, Formal analysis, Validation, Writing – review & editing.

F.J. Peña: Data curation, Methodology, Funding acquisition, Supervision, Formal analysis, Validation, Writing – review & editing.

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Mario Estévez: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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5. DISCUSIÓN

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5.1 Efecto de los productos de oxidación de lípidos (MDA) y de proteínas (AAA) en *L. reuteri*

Uno de los objetivos de la Tesis es dilucidar el impacto que diversos productos de oxidación de lípidos y proteínas presentes en alimentos procesados pueden tener sobre bacterias probióticas. Para cumplir con este objetivo, una cepa de *Lactobacillus reuteri* PL503 aislada a partir de heces de cerdo fue expuesta al efecto de dos productos finales de la oxidación lipídica y proteica, el malondialdehído (MDA) y el ácido α-aminoadípico (AAA), respectivamente. La incubación de *L. reuteri* con los compuestos derivados de la oxidación de lípidos, MDA (0, 5, 25 Y 100 µM) (Capítulo 4.I), y de proteínas, AAA (0, 1, 5 y 10 mM) (Capítulo 4. III), no afectó a la supervivencia de la bacteria, lo que implica que los rangos de concentración utilizados no provocaron cambios letales, pero sí indujo la puesta en marcha de mecanismos bacterianos para neutralizar los efectos perjudiciales de estos productos de oxidación. A pesar de estas respuestas génicas y bioquímicas que buscan hacer frente al efecto potencialmente nocivo de los productos de oxidación, éstas no fueron suficientes para contrarrestar el impacto de estas sustancias sobre la biología de la bacteria. En los siguientes apartados se comparan los efectos de ambas sustancias sobre la respuesta de *L. reuteri* así como la severidad del daño que ambos compuestos finalmente causaron a esta bacteria.

5.1.1. Efecto sobre la expresión génica

La incubación de L. reuteri PL503 con los productos de oxidación en estudio (MDA y AAA) tuvo diferente efecto sobre la expresión de los genes analizados. Como ya se describió en el capitulo I de esta Tesis Doctoral, el gen uspA codifica para una proteína de la superfamilia universal stress protein A, que es un grupo de proteínas conservadas que se encuentran en microorganismos, insectos y plantas y, aunque el mecanismo de acción de la proteína no está claro, parece que tiene que ver con la defensa contra agentes que dañan el ADN (Kvint et al., 2003).

En el análisis de la expresión del gen *uspA* incubado en presencia de MDA se detectó , en general, una sobreexpresión . Estos mismos resultados se observaron en un estudio con *Lactobacillus fermentum* bajo diferentes condiciones de estrés (Kaur et al., 2017). Aunque no se conoce el mecanismo de accion del gen *uspA*, se sabe que puede estar implicado en la reparación del ADN. Este hecho tiene gran relevancia en el caso del MDA ya que éste reacciona con el ADN, formando aductos como la pirimidopurinona (M1G) que se ha vinculado a la aparición de cáncer asociado a la dieta en humanos (Niedernhofer et al., 2003), y que en bacterias da lugar a mutagénesis (Marnett, 1999). Es, precisamente, esta amenaza de mutagénesis la que puede haber llevado a la sobreexpresión que se obtuvo en el gen *uspA*.

Sin embargo, en presencia de AAA, se observó una inhibición del gen *uspA* en la primera mitad del ensayo (12 y 16 h de incubación), que coincide con los resultados obtenidos por Oberg et al. (2015), en un estudio con *B. longum* incubado en un sistema de generación de radicales y por Arcanjo et al. (2019) *en L. reuteri* frente a 0,5 mM de H_2O_2 donde también se observó una disminución en la expresión del mismo gen. La bacteria reacciona de la misma manera frente al AAA y frente al sistema de generación de radicales mencionado, lo que implica que la molécula en estudio es reconocida como una amenaza. Esto ha sido confirmado mediante estudios recientes en células intestinales (Díaz-Velasco et al., 2020) y pancreáticas (Estaras et al., 2020) cultivadas con AAA en concentraciones compatibles con los alimentos (200 µM), y donde se ha observado la capacidad del aminoácido oxidado para inducir estrés oxidativo y apoptosis en células humanas. Por ello, la inhibición de este gen puede estar relacionada con la señalización celular inducida por el ambiente prooxidante.

La diferencia de la expresión del gen *uspA* en *L. reuteri* bajo los dos compuestos estudiados puede ser debida a la especificidad de la respuesta génica, ya que cada uno de los compuestos produce un daño diferente sobre la bacteria. El MDA afecta al ADN, por lo que se aumenta la expresión de este gen, ya que es la función de esta proteína, y el AAA activa mecanismos de señalización celular que dan respuesta a una amenaza prooxidante.

Por otro lado, el gen *dhaT* codifica para la enzima 1,3-PDO, que tiene un papel importante en situaciones de estrés con necesidad de energía. Esta enzima facilita la principal ruta de fermentación (6-fosfogluconato/fosfoketolasa; 6 PG/PK) a través de la producción de NAD^+ (que se necesita para la fermentación de la glucosa) desde NADH en la conversión de 3-hidroxipropionaldehído (3-HPA) a 1,3-propanodiol (1,3-PD) en condiciones anaeróbicas (Schaefer et al., 2010).

La respuesta de este gen frente a los dos compuestos es similar, ya que en ambos casos se produce una sobreexpresión del mismo a tiempos finales de incubación, que puede responder a un intento de la bacteria de protegerse del daño oxidativo mediado por el producto de oxidacion de lípidos y por el aminoácido oxidado. Estos resultados concuerdan con los de Arcanjo et al. (2019), que estudiaron la respuesta de *L. reuteri* incubado con H_2O_2.

El mecanismo mediante el que la bacteria elimina el daño inducido por los compuestos en estudio, pasa por la activación de la ruta del 3-HPA, que tiene como precursor al glicerol (Figura 5.1; Arcanjo et al., 2019), ausente en estas condiciones de cultivo, por lo que esta ruta es poco probable. Ya que el principal precursor no está disponible, es lógico pensar que 1,3-PDO tiene otros sustratos y que su función puede estar relacionada con la protección frente a daño oxidativo. Arcanjo et al. (2019) propusieron que la actividad de 1,3-PDO dependiente de NAD^+ eliminaba el H_2O_2 en presencia de NADH

(Figura 5.1). En los ensayos de la presente Tesis Doctoral el H_2O_2 no estaba presente, por lo que la implicación del gen *dhaT* en el equilibrio del estado redox parece ser un mecanismo de defensa frente a los compuestos prooxidantes estudiados.

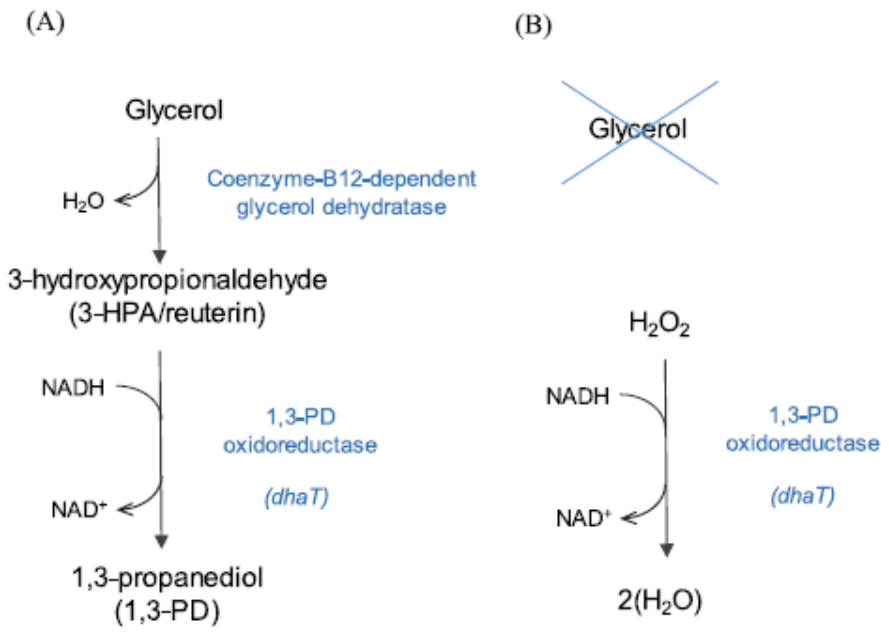

Figura 5.1: (A) Producción de 3-HPA y 1,3-PD desde glicerol en *Lactobacillus reuteri* según lo desccrito por Talarico et al. (1990) y (B) mecanismo propuesto por Arcanjo et al. (2019) por el que la NADH-oxidorreductasa codificada por el gen *dhaT* parece detoxificar el H_2O_2 (Fuente: Arcanjo et al., 2019).

Durante la primera mitad de ambos ensayos (12 y 16 h de incubación), se observa una inhibición del gen *dhaT* que puede ser debida a las fases inicales de respuestas de la bacteria frente al estrés, donde en ese momento no es necesaria la acción de la proteína codificada por el gen *dhaT*.

Sin embargo, durante la segunda mitad del ensayo (18-24 h de incubación), el avanzado estado de estrés oxidativo producido por los dos compuestos añadidos, hace que la expresión del gen *dhaT* aumente, desencadenando la respuesta de defensa antioxidante. Si bien, ignoramos en qué medida estos compuestos pueden estar implicados en la generación de radicales libres y otras sustancias prooxidantes (H_2O_2), resulta evidente, a la vista de los resultados de la citometría de flujo y daño oxidativo bacteriano descritos posteriormente (Capítulo 5.1.2) que ambas sustancias alteran el estado redox de la bacteria lo que concuerda con una mayor expresión de un gen que codifica para una enzima oxidorreductasa dependiente de NADH.

Si bien los mecanismos de acción de los dos compuestos analizados merecen ser estudiados de forma más precisa mediante, por ejemplo, técnicas ÓMICAS (como la transcritpómica o la proteómica),

podemos afirmar que ambas producen daño oxidativo en la bacteria, y esto hace que se activen las respuestas de defensa antioxidante con la sobreexpresión del gen *dhaT*, entre otros mecanismos, implicado en las respuestas a estrés.

5.1.2. Efecto sobre la generación de radicales

El estudio de la acumulación de radicales libres se ha realizado mediante citometría de flujo con ambos compuestos (MDA y AAA), siendo los resultados coincidentes en el efecto dosis que se observa: a mayor concentración, mayor es la capacidad del compuesto de generar estrés oxidativo en la bacteria, que se traduce en mayor concentración de bacterias con ROS.

En la bacteria incubada con MDA se obtienen valores del CellRox+ de entre el 0 y el 8,16%, mientras que con AAA se obtienen entre 0,8 y 5,3% lo que indica que los dos compuestos inducen estrés oxidativo con la presencia de radicales libres en las bacterias. Si bien la conexión es evidente y el efecto dosis-respuesta indica una probable causalidad, los mecanismos moleculares implicados en la generación de dichos radicales son desconocidos.

En el caso del MDA, es posible que, al ser un potente aceptor de electrones, pueda favorecer la formación de H_2O_2, ya que otras sustancias de similares características (aceptores de electrones como O_2 y fructosa) han demostrado ser capaces de generar dicho compuesto prooxidante (Mane, 2016).

Por otro lado, aunque no hay estudios previos realizados en bacterias incubadas con AAA, Díaz-Velasco et al. (2020), en estudios con células CACO-2, y Estaras et al. (2020), en células pancreáticas expuestas a AAA, observaron que se produce un desequilibrio en el estado redox, la generación de ROS, la apoptosis y necrosis celular.

5.1.3. Daño oxidativo sobre proteínas y lípidos bacterianos

En el estudio de las proteínas y lípidos bacterianos, se pudo confirmar que la generación de radicales libres en la bacteria causó estrés oxidativo medido a través del daño oxidativo en las estructuras bacterianas, en concreto, lípidos y proteínas. El daño oxidativo en los lípidos se midió a través de la cuantificación de MDA y otras sustancias reactivas al TBA (TBARS) mientras que la oxidación proteica se midió a través de la cuantificación del aminoácido oxidado, el AAS, también conocido como alisina, mediante cromatografía líquida. Este compuesto es el principal carbonilo en sistemas biológicos (Stadtman & Levine, 2000) y se ha propuesto como indicador de senescencia en bacterias (Ezraty et al., 2017).

Teniendo en consideración que la sustancia que se utilizó en uno de los experimentos (MDA) es justo uno de los principales productos de oxidación de los lípidos (Capítulo 4.I), su detección y evolución en las bacterias durante el ensayo no nos sirvió tanto para evaluar el daño oxidativo sobre la bacteria (la cantidad de MDA añadida es muy superior a la que se podría generar *de novo* en la bacteria como consecuencia del estrés oxidativo), sino como para detectar en qué medida la bacteria es capaz o no de detoxificar dicha sustancia. En este estudio (Capítulo 4. I) se observó que i) la concentración inicial de TBARS medido en cada grupo experimental era coherente con la cantidad de MDA añadida y ii) que a medida que avanzó el tiempo de incubación se redujo la cantidad de TBARS entre el 22 y el 26%, lo que puede ser debido a las reacciones producidas entre el MDA y otras biomoléculas, incluido el ADN, con lo que también se podría explicar la sobreexpresión de los dos genes estudiados a tiempos finales de incubación (Capítulo 5.1.1). Otra hipótesis es que el MDA pudiera reaccionar con las proteínas, contribuyendo a la formación de carbonilos. De hecho, la concentración del principal carbonilo en proteínas (el anteriormente mencionado AAS) se incrementó de forma efecto-dosis en bacterias expuestas a concentraciones crecientes de MDA (hasta los 3 nmol/mg proteína). En esta Tesis Doctoral es la primera vez que se utiliza la determinación de alisina como marcador de oxidación proteica en bacterias, de la que una cantidad mayor a 1 nmol de carbonilo por mg de proteína da lugar a condiciones de estrés oxidativo (Akagawa et al., 2002).

Con el objeto de dilucidar los mecanismos implicados en la generación de carbonilos de proteínas a partir de la reacción de éstas con el MDA, se planteó específicamente un nuevo trabajo (Capítulo 4.II), en el que dicho compuesto se incubó con diferentes tipos de proteínas, tanto de alimentos como humanas. La alisina se forma como resultado de la desaminación oxidativa del grupo ε-amino en residuos de lisina, y esta oxidación puede ser iniciada por radicales libres (Utrera & Estévez, 2012) o por dicarbonilos que provienen de la reacción de Maillard (Akagawa et al., 2002). Resulta razonable hipotetizar en qué medida dicarbonilos procedentes de la oxidación lipídica (MDA) son capaces de generar carbonilos en proteínas mediante un mecanismo similar al de los dicarbonilos procedentes de la reacción de Maillard (como por ejemplo el glioxal o el metilglioxal) (Arcanjo et al., 2018). Como resultado se observó que la concentración de alisina no cambió en la suspensión de proteínas con MDA y, por lo tanto, este compuesto no induce la desaminación oxidativa de los residuos de lisina, como se había señalado previamente. Sin embargo, la reacción del MDA con los aminoácidos alcalinos generó uniones covalentes (adición de Michael) estables entre uno de los aldehídos del MDA y el grupo amino de la cadena lateral de la lisina y otros aminoácidos alcalinos, lo que provocó la unión de moléculas de MDA en la superficie de las proteínas (Capítulo 4.II). Si bien, esta reacción descarta la capacidad del MDA de inducir la desaminación oxidativa de la lisina y la formación de alisina (carbonilo primario),

esta unión es un proceso de carbonilación en sí mismo (carbonilación secundaria: introducción de carbonilos de lípidos en las proteínas).

Los resultados del Capítulo 4.II permite explicar la "disminución" de la concentración de MDA en los medios con bacterias (Capítulo 4.I), ya que se estarían uniendo efectivamente a las proteínas, entre, probablemente, otras biomoléculas. Por otro lado, indican que el mecanismo más probable de la inducción de carbonilación primaria (formación de alisina) en las bacterias tratadas con MDA es mediante la generación de radicales libres que son los que finalmente causan la desaminación de residuos de lisina. El mecanismo implica la formación de H_2O_2 y su descomposición por la reacción de Fenton (Figura 5.2) dando lugar a radicales hidroxilo, que participarían en la desaminación oxidativa mediada por radicales de los residuos de lisina como el mecanismo más probable para la formación de los carbonilos de proteínas.

$$M^{n} + H_2O_2 \rightarrow M^{n+1} + HO^{\bullet} + HO^{-}$$

Figura 5.2: Reacción de Fenton. Donde M es un metal de transición, HO- es el ion hidroxilo y HO• es el radical hidroxilo, el radical libre más abundante y peligroso en los sistemas biológicos (Fuente: Davies, 2005).

La carbonilación de proteínas, descrita por primera vez en bacterias a través de la detección y cuantificación de un carbonilo primario específico (la alisina), es una modificación irreversible de las proteínas en ambientes prooxidantes y suele tener consecuencias biológicas negativas. Las proteínas carboniladas pueden tener dos destinos:

- Ser eliminadas, ya que su acumulación puede causar un desequilibrio celular, que da lugar a alteraciones crónicas o apoptosis (Shacter, 2000).
- Funcionar como moléculas señal, que activan rutas específicas para controlar la homeostasis (Shacter, 2000).

Aún es necesario investigar con mayor detalle el papel que estos carbonilos podrían jugar en bacterias aunque tal y como indicó Ezraty et al. (2017), y se propone en la presente Tesis Doctoral, podrían indican tanto daño oxidativo y estar relacionado con la senescencia bacteriana, como conducir a mecanismos de señalización encaminados a responder ante dicha situación de estrés, mediante, por ejemplo, la expresión génica.

En la incubación con AAA (Capítulo 4.III), se observa una disminución de TBARS respecto al control en la segunda mitad del ensayo. Sin embargo, entre las concentraciones utilizadas no aparecieron diferencias significativas. Este aminoácido oxidado no parece inducir de forma significativa peroxidación lipídica en muestras de *L. reuteri* a pesar de su probada capacidad para generar radicales libres. Por el contrario, se observó la inducción de oxidación proteica en bacterias incubadas con AAA. En coherencia con los resultados obtenidos en el Capítulo 4.I, la incubación de *L. reuteri* con MDA y con AAA conduce a la carbonilación de proteínas, en concentraciones superiores a 3 nmol o 10 nmol (respectivamente). Al igual que en el caso del MDA, la formación de carbonilos primarios (alisina) en proteínas bacterianas debe responder a la generación de radicales libres que terminan ejecutando la desaminación oxidativa de residuos de lisina. Este fenómeno de carbonilación primaria, coincide, en el tiempo, con una sobreexpresión del gen *dhaT* por lo que se ha propuesto, de acuerdo con Arcanjo et al. (2019) y Ezraty et al. (2017), que el mecanismo de activación de este gen se da por H_2O_2 o por el efecto de la carbonilación de proteínas, dando lugar a respuestas antioxidantes, como, en este caso, la expresión de un gen que codifica una enzima con capacidad oxidoreductasa.

Según los datos obtenidos en los ensayos realizados (Tabla 5.1), parece que el AAA tiene un efecto más intenso sobre la oxidación de proteínas, ya que se observan valores de alisina por encima de 11 nmol de alisina por mg de proteína, siendo la mayor cantidad encontrada en MDA de 5 nmol/mg. En la Tabla 5.1 además, se resume el efecto de ambas sustancias sobre la expresión génica y la formación de ROS medida por citometría de flujo Capítulos 5.1.1 y 5.1.2., respectivamente).

Tabla 5.1: Tabla resumen de los efectos producidos por MDA y AAA sobre *Lactobacillus reuteri.*

Producto de oxidación	**Expresión génica**		**Formación de ROS**	**Daño oxidativo**	
	uspA	***dhaT***		**Lípidos**	**Proteínas**
Malondialdehído	+	+	+	+	+
Ácido aminoadípico	-	+/-	+	-	++

Expresión génica: + sobreexpresión; - inhibición. Formación de ROS: + acumulación de ROS. Daño oxidativo: lípidos: + oxidación, - no significativo; proteínas: +: [alisina] > 4 nmol/mg, ++: [alisina] > 10 nmol/mg

5. DISCUSIÓN

5.2. Efecto del ácido clorogénico sobre el estrés oxidativo producido por H_2O_2 en *E. faecium*

Una vez estudiado el efecto de productos de oxidación específicos sobre la aparición de estrés oxidativo en una bacteria probiótica (*L. reuteri*) y las respuestas de ésta en términos de expresión génica, se planteó como nuevo objetivo analizar en qué medida la adición de un compuesto fenólico muy conocido, abundante y caracterizado AC (500 µM) tiene efecto sobre el estrés oxidativo en otra bacteria con potencial probiótico (*E. faecium*). En este caso y a diferencia de los trabajos anteriores, la inducción de estrés oxidativo se llevó a cabo mediante un sistema generador de radicales libres a través de la exposición a H_2O_2 (2,5 mM) y la reacción de Fenton.

En esta discusión conjunta, en primer lugar, se comparará el efecto pro-oxidante de este sistema (Capítulo 4.IV) con el estrés producido por el MDA (Capítulo 4.I) y el AAA (Capítulo 4.III). Posteriormente, se evaluará el efecto del AC sobre dicha situación de estrés mediante el uso de técnicas avanzadas como la proteómica.

5.2.1. H_2O_2 *vs* MDA y AAA como inductores de estrés oxidativo

En el ensayo realizado con *E. faecium* (Capítulo 4.IV), la capacidad del H_2O_2 para producir ROS en la bacteria se determinó, al igual que en los ensayos previos, mediante citometría de flujo. Se observó, como en los casos anteriores, un incremento en la cantidad de bacterias CellRox+ (bacterias con radicales libres) respecto al control. Mediante esta técnica se ha visto que el H_2O_2 afecta a la generación de radicales libres en la bacteria, lo que concuerda con los resultados obtenidos por Manoil & Bouillaguet, (2018) quienes evaluaron, en *E. faecalis* y *Fusobacterium nucleatum* la capacidad del H_2O_2 de producir estrés oxidativo mediante citometría de flujo. La descomposición del compuesto probablemente se produce por la reacción de Fenton (Figura 5.2), utilizando metales presentes en la composición del medio de cultivo que favorecen esta reacción, con la consiguiente acumulación de radicales libres.

Comparando el efecto del H_2O_2 con los otros agentes prooxidantes estudiados (MDA y AAA), se observa que el efecto ocasionado sobre la producción de radicales libres en *E. faecium* por el H_2O_2 es similar al causado por los productos de oxidación de lípidos (MDA) y proteínas (AAA) en *L. reuteri*. De la misma manera que ocurrió con estos últimos, la incubación con H_2O_2 produjo daño oxidativo en lípidos y proteínas de la bacteria. En cuanto a la formación de carbonilos, en la incubación con H_2O_2 se obtuvieron mayores concentraciones de alisina, cuantificándose 15 nmol de carbonilo/mg de proteína, cantidad superior a las mayores observadas con MDA (5 nmol carbonilo/mg de proteína) y con AAA

(11,7 nmol carbonilo/mg de proteína). Sin embargo, en el daño oxidativo producido en lípidos, se observa mayor oxidación en AAA (1,17 mg TBARS/L), que en H_2O_2 donde se observa 0,7 mg TBARS/L.

Mediante las determinaciones realizadas en esta Tesis Doctoral se pone de manifiesto, de manera original, que los productos de oxidación presentes en alimentos producen daño oxidativo en bacterias probióticas, al igual que los radicales libres. Por ello, el consumo de alimentos procesados, ricos en productos de oxidación lipídica y proteica, conduce a la aparición de disbiosis, es decir, la alteración del equilibrio de la microbiota, caracterizada por un descenso de las bacterias protectoras y un aumento de bacterias con efectos negativos, lo que está de acuerdo con otros estudios en carne y productos cárnicos que concluyen, de forma similar, que los productos de oxidación producen la mencionada disbiosis microbiana (Estévez & Xiong, 2019; Macho-González et al., 2020).

En definitiva, los compuestos derivados de alimentos procesados (MDA y AAA), alteran la la microbiota intestinal, que se relaciona con diversas patologías del tracto gastrintestinal. La disbiosis causada por los productos de oxidación podría estar contribuyendo a la aparición de un ambiente favorecedor para el desarrollo de procesos proinflamatorios (Losso, 2021; Wang et al., 2021)

5.2.2. Efecto del ácido clorogénico sobre el estrés oxidativo en *E. faecium*

El AC es un compuesto fenólico, que pertenece a la familia de los ácidos hidroxicinámicos, se encuentra en frutas y verduras como las manzanas, granos de café, el té y la alcachofa; y tiene efectos antioxidantes, antiinflamatorios, antidiabéticos o antihipertensivos (Santana-Gálvez et al., 2017). En esta Tesis Doctoral se ha estudiado el efecto que este compuesto tiene sobre *E. faecium* en su defensa frente al efecto pro-oxidante del H_2O_2.

En los resultados de citometría de flujo se observa que el AC por sí mismo tiene efectos prooxidantes sobre la bacteria, también observados en otros sistemas biológicos (Hou et al., 2017) y en bacterias (Wang et al., 2020). Por lo tanto, el AC es capaz de inducir estrés oxidativo (alrededor del 8% de de células CellRox+), leve pero significativo, en un mayor número de bacterias que el H_2O_2 (5%), ya que la cantidad de ROS en los grupos con AC (intensidad de fluorescencia 5) es mucho menor que en H_2O_2 (intensidad de fluorescencia 7).

Sin embargo, en la combinación de AC con H_2O_2, hay menor cantidad de bacterias con ROS (3% CellRox+), aunque en ellas la fluorescencia detectada es mayor (intensidad de fluorescencia 11), teniendo por tanto los mayores niveles de radicales. Esto quiere decir que el AC, en comparación con el grupo tratado solo con H_2O_2, disminuye el número de células con ROS, lo que demuestra el efecto

protector del compuesto fenólico frente a la propagación del estrés oxidativo, aunque las bacterias que sí sufren este estrés tienen grandes cantidades de ROS.

Por otro lado, la peroxidación de lípidos en *E. faecium* se redujo con la presencia de AC, tanto solo (0,45 mg TBARS/ L) como cuando se combinó con H_2O_2 (0,4 mg TBARS/ L). Tal y como se discute en el Capítulo 4.IV, es razonable considerar que el limitado estrés oxidativo causado por el AC llevara a la activación de defensas antioxidantes que, finalmente, protegieran a los lípidos bacterianos frente a la oxidación. Arcanjo et al., (2019) obtuvieron resultados similares en la incubación de *L. reuteri* con resveratrol, un compuesto fenólico antioxidante, en presencia de H_2O_2.

Sin embargo, la presencia de AC generó un aumento importante de la cantidad de carbonilos de proteínas (18 nmol carbonilo/mg proteína), que podría estar asociado con la conocida actividad prooxidante de su forma quinona (Utrera & Estévez, 2012). Además, se sabe que la carbonilación de proteínas puede ser un mecanismo de señalización celular, mediante el que se activen las respuestas antioxidantes con el que la bacteria tiene la capacidad de degradar el H_2O_2. De hecho, la actividad tipo-catalasa de esta bacteria se incrementó considerablemente en las bacterias expuestas a la combinación de AC con H_2O_2 (25 pmol H_2O_2/min*mL), lo que proporciona fortaleza a nuestra hipótesis.

En conclusión, el AC genera cierto estrés oxidativo en la bacteria, lo suficiente como para que se activen respuestas de defensa y en combinación con H_2O_2 se produce una cierta protección de la bacteria. De este modo, el AC podría estar actuando como prebiótico, mejorando la disposición de *E. faecium* para hacer frente a la amenaza prooxidante del H_2O_2 y para ello actúa sobre la carbonilación de las proteínas bacterianas que podrían, a su vez, jugar un papel como señalización celular (indicando la presencia de dicha amenaza prooxidante) que favorece la probable síntesis y actividad de enzimas antioxidantes. Este tipo de mecanismo ya se describió en los Capítulos 4.I y 4.III, donde la acumulación de alisina (carbonilo primario) en proteínas bacterianas, se propuso como detonante de la respuesta antioxidante de *L. reuteri* a través de la expresión del gen *dhaT* que codifica una oxidorreductasa con efectos protectores frente al estrés oxidativo. En estudios recientes en los que se evalúan los mecanismos moleculares de *Lactobacillus*, incubado con diversos compuestos fenólicos de plantas, como el resveratrol o el ácido gálico, se ha visto que se induce su respuesta antioxidante (López de Felipe et al., 2022).

5.3. Progreso científico en el conocimiento de la biología bacteriana mediante el uso de métodos avanzados: expresión génica, citometría de flujo y proteómica no dirigida.

5.3.1. Expresión génica

El análisis de la expresión génica mediante qPCR es una herramienta valiosa y altamente específica para comprender la influencia de los factores externos sobre determinadas vías y funciones metabólicas de las bacterias en un momento dado (Taniguchi et al., 2009).

Sin embargo, esta técnica puede suponer un desafío en el laboratorio, ya que es necesario que las preparaciones de ARN sean de alta calidad, libres de contaminantes y no degradadas, ya que la desnaturalización del ARN se produce fácilmente. Además, la puesta a punto de las condiciones de reacción es compleja, debido a que para cada conjunto de cebadores es necesario estimar la eficiencia de la reacción utilizando una curva estándar, así como el análisis de las curvas de disociación; las condiciones de trabajo deben ser muy estrictas y se requiere una inversión inicial importante.

El estudio de la expresión de los genes relacionados con el estrés (*uspA* y *dhaT*) implicados en la respuesta de las bacterias probióticas a situaciones de oxidantes causado por distintos agentes (AAA, MDA, H_2O_2) nos ha permitido conocer:

- el efecto de estos compuestos sobre la bacteria,
- la implicación que tienen estos genes frente al estrés oxidativo inducido,
- las posibles rutas de respuesta bacteriana a las diferentes a situaciones estrés.

Todo ello puede estar asociado a un efecto beneficioso de la bacteria en el hospedador, ya que al protegerse de esa amenaza puede proteger al hospedador. Si bien este mecanismo de efecto probiótico debería ser probado en un sistema *in vivo* en futuros trabajos.

El estudio del transcriptoma de bacterias probióticas en condiciones de estrés oxidativo permite identificar genes sobreexpresados o inhibidos en presencia de nuevos compuestos prooxidantes (Oberg et al., 2015), lo que puede facilitar próximos estudios de los efectos sobre la microbiota en la defensa contra el estrés oxidativo generado en el lumen intestinal. De confirmarse, tanto el efecto negativo de la ingesta de productos de oxidación sobre la microbiota beneficiosa, como la posible protección que ésta ofrece frente al estrés oxidativo luminal, se deberían desarrollar estrategias antioxidantes dietéticas, encaminadas a combinar de forma efectiva la ingesta de bacterias probióticas

con alimentos de origen vegetal ricos en compuestos fenólicos, como el ácido clorogénico, entre muchos otros.

5.3.2. Citometría de flujo

La citometría de flujo es una herramienta rápida, sencilla, sensible y económica con la que se obtiene información de varios parámetros como la fisiología, morfología o genética de las células en estudio mediante la utilización de diversas sondas (Steen, 2000). Sin embargo, presenta desventajas como la inversión inicial en el citómetro, la puesta a punto de la concentración de sonda para evitar artefactos no deseados y la concentración de bacteria a utilizar (Cossarizza et al., 2017).

El uso de sondas específicas para ROS que son identificadas por citometría de flujo ofrecen una alta sensibilidad (DeLoughery et al., 2014). Las sondas utilizadas en esta Tesis Soctoral son: i) CellROX Deep Red que entra en el citoplasma de la célula y emite fluorescencia brillante tras su oxidación en presencia de ROS; y ii) Hoechst 33342, que marca el ADN bacteriano permitiendo la identificación de células viables.

La sonda CellROX Deep Red ha sido normalmente utilizada en células de mamífero (Manoil & Bouillaguet, 2018). Sin embargo, estudios recientes, entre los que se encuentran los de esta Tesis Doctoral, han demostrado que este reactivo puede ser utilizado para detectar estrés oxidativo en bacterias. Concretamente, Manoil & Bouillaguet (2018) lo han utilizado para identificar la producción de ROS en *E. faecalis* y *F. nucleatum*. Además, Parbhoo et al. (2020) han utilizado esta sonda en el desarrollo y la aplicación de la citometría de flujo para el avance del conocimiento de la fisiología y patogénesis de *Mycobacterium tuberculosis*, mediante el estudio de los factores de estrés asociados al huésped que influyen sobre las características bacterianas (actividad metabólica, potencial de membrana, estado redox y la pared celular bacteriana).

El futuro de la citometría de flujo pasa por aplicar estudios multiparamétricos de próxima generación (avanzados), como los que se han utilizado en este estudio. Sin embargo, otras combinaciones de sondas podrían arrojar nueva información en el estudio de estrés oxidativo en bacterias. Por ejemplo, la utilización de dos sondas (CellROX Green y Yoduro de propidio) para estudiar el estrés oxidativo en bacterias probióticas, podrían mejorar la descripción del ciclo de vida de las ROS, al discriminar entre las etapas de formación, acumulación y agotamiento de las mismas (Fallico et al., 2020).

En esta Tesis Doctoral, gracias a esta técnica, se han clasificado las bacterias en función de su estado redox, se ha identificado la generación de radicales libres y se ha observado el efecto que producen MDA, AAA y H_2O_2 en la inducción de estrés oxidativo en las poblaciones bacterianas estudiadas. Hasta

la fecha no existen otros estudios que utilicen la citometría de flujo para evaluar la capacidad de dichos compuestos químicos de producir estrés oxidativo sobre bacterias probióticas. Por lo tanto, la contribución de los resultados y conclusiones de esta Tesis Doctoral puede facilitar la aplicación de esta técnica en estudios posteriores.

5.3.3. Proteómica no dirigida

La proteómica es el estudio del conjunto de proteínas que forman un organismo, sus interacciones y las funciones que realizan (Dutt & Lee, 2000). Es una técnica muy sensible, que sirve tanto para identificar, como para cuantificar proteínas presentes en células eucariotas, así como en bacterias. La aplicación de esta técnica en bacterias se encuentra mucho menos desarrollada que en células eucariotas (animales o vegetales). En cualquier caso, en la bibliografía científica se describen varias aplicaciones entre las que destaca la investigación de los cambios producidos en procesos redox (Allan Butterfield & Dalle-Donne, 2012) o la evaluación de la seguridad de microorganismos probióticos (Cirrincione et al., 2019).

Uno de los pasos limitantes del estudio del proteoma de bacterias es la extracción de proteínas, ya que de ésta dependen la sensibilidad y el rendimiento de los pasos posteriores. En el estudio del proteoma *de E. faecium* se llevó a cabo la puesta a punto del método, siendo la extracción de proteínas uno de los desafíos más complejos planteados, junto con la posterior lisis proteica. La información obtenida de la bacteria mediante esta técnica da a conocer el estado fisiológico de la misma, así como la respuesta de la bacteria frente a cualquier estímulo externo, como en esta Tesis Doctoral, agentes prooxidantes o compuestos fenólicos.

Entre las aplicaciones futuras de esta técnica avanzada, partiendo del conocimiento obtenido en esta Tesis Doctoral pueden estar los estudios de bacterias probióticas bajo diferentes condiciones de estrés provocado por otros agentes prooxidantes, así como la comparación de estos con la incubación con otros agentes antioxidantes, para descifrar las rutas biológicas implicadas.

En la Tabla 5.2 se resumen las ventajas y dificultades, así como los desafíos futuros asociados a las técnicas moleculares avanzadas aplicadas en esta Tesis Doctoral.

Tabla 5.2: Resumen técnicas moleculares avanzadas

Método	Fortalezas/Ventajas	Dificultades	Desafíos futuros
Expresión génica	- Cuantificación de la expresión de genes en un momento y ambiente concreto del crecimiento bacteriano. - Elección de genes diana de estrés oxidativo y endógeno.	- Integridad del ARNm, por su facilidad de desnaturalización. - Puesta a punto de las condiciones de reacción.	- Estudio del transcriptoma de bacterias probióticas en condiciones de estrés oxidativo
Proteómica	- Composición de las proteínas de la bacteria en un momento concreto	- Extracción de proteínas	- Estudio del proteoma de la bacteria en otras condiciones de estrés oxidativo
Citometría de flujo	- Rápida - Sencilla, -Sensible - Económica	- Necesidad de puesta a punto de la concentración de bacterias y sondas	- Nuevas sondas para la aplicación en estudios de estrés oxidativo en bacterias

6. CONCLUSIONES

6. CONCLUSIONES

1. El principal producto de oxidación de lípidos en alimentos, el MDA, es reconocido por la bacteria probiótica *L. reuteri* como un producto potencialmente tóxico que induce la formación de radicales libres y estrés oxidativo que afecta tanto a lípidos como a proteínas bacterianas.

2. La exposición de *L. reuteri* a AAA, producto de oxidación de la lisina, conduce a un incremento en la formación de radicales libres con particular afectación de la oxidación de proteínas bacterianas.

3. A dosis compatibles con alimentos, MDA y AAA no comprometen la supervivencia de *L. reuteri,* que pone en marcha mecanismos de defensa antioxidante para mantener su estado redox y homeostasis.

4. *L. reuteri* responde específicamente a la amenaza causada por MDA y AAA: mientras que el MDA induce activación del gen *uspA*, protector frente al estrés y la reparación del ADN bacteriano, el AAA lo inactiva.

5. MDA y AAA activan el gen *dhaT* de *L. reuteri*, el cual codifica una enzima oxidorreductasa considerada clave en su defensa frente a alteraciones del estado redox. Se propone además que la carbonilación de las proteínas bacterianas podría tener un papel de señalización que activa los mecanismos de defensa antioxidante.

6. Se propone que los mecanismos de defensa antioxidante que *L. reuteri* pone en marcha y que conducen, entre otros resultados, a la detoxificación del MDA y de otras sustancias prooxidantes, podrían proteger al hospedador, y por lo tanto, alguno de los mecanismos moleculares descritos en la presente Tesis Doctoral podrían explicar el beneficio que dicha bacteria tiene frente a alteraciones del tracto gastrointestinal.

7. Frente a un agente oxidante como el peróxido de hidrógeno, *E. faecium* reduce sus rutas metabólicas de producción de energía y refuerza sus paredes y membranas celulares, lo que indica que la bacteria es capaz de responder a condiciones desfavorables y defenderse de ellas.

8. El ácido clorogénico tiene un efecto protector en *E. faecium* ya que refuerza las defensas antioxidantes endógenas mediante la activación de rutas de señalización celular probablemente mediadas por procesos de carbonilación proteica.

9. Las técnicas moleculares empleadas (expresión génica, citometría de flujo y proteómica) son valiosas para profundizar, a nivel molecular, en el conocimiento de la biología bacteriana y los posibles efectos beneficiosos de bacterias probióticas.

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